Ecotoxicological evaluation of treated wastewater and sewage sludge for sustainable use in land management

Dissertation

zur Erlangung des Doktorgrades

der Naturwissenschaften

vorgelegt beim Fachbereich Biowissenschaften

der Johann Wolfgang Goethe-Universität

in Frankfurt am Main

von

Elisabeth Richter

aus Berlin

Frankfurt 2015

(D30)

vom Fachbereich Biowissenschaften der
Johann Wolfgang Goethe-Universität als Dissertation angenommen.

Dekanin:
Prof. Dr. Maike Piepenbring
Johann Wolfgang Goethe-Universität Frankfurt am Main
Institut für Ökologie, Evolution und Diversität
Mykologie
Max-von-Laue-Str. 13, D-60438 Frankfurt am Main

Gutachter:
Prof. Dr. Jörg Oehlmann
Johann Wolfgang Goethe-Universität Frankfurt am Main
Institut für Ökologie, Evolution und Diversität
Aquatische Ökotoxikologie
Max-von-Laue-Str. 13, D-60438 Frankfurt am Main

Prof. Dr. Thomas A. Ternes
Bundesanstalt für Gewässerkunde
Referat G2
Am Mainzer Tor 1, D-56068 Koblenz

Datum der Disputation: 24. Februar 2016

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Abbreviations

AbfKlärV	Klärschlammverordnung
AbwV	Abwasserverordnung
BBodSchG	Bundesbodenschutzgesetz
BBodSchV	Bundesbodenschutzverordnung
BCF	bioconcentration factor
BDDA	benzyldimethyldodecylammonium chloride
CEN	Comité Européen de Normalisation
CYP	cytochrome
d	day
d.w.	dry weight
DDT	dichlorodiphenyltrichloroethane
DüMV	Düngemittelverordnung
EC	European Commission
EC_{50}	median effective concentration
EEC	European Economic Community
EG	Europäische Gemeinschaft
EMEA, since 2009 EMA	European Medicines Agency
EOX	extractable organic halogens
EQS	Environmental quality standard
EU	European Union
h	hour
ha	hectar
ISO	International Organization for Standardization,
kg	kilogram
K_{oc}	Soil Organic Carbon-Water Partitioning Coefficient
L	liter

LASs	linear alkyl sulfonates
MEC	measured environmental concentration
mg	milligram
µg	microgram
mio	million
ng	nanogram
NPEs	nonylphenol ethoxylates
OC	organic carbon
PAHs	polyaromatic hydrocarbons
PCBs	polychlorinated biphenyls
PEC	predicted environmental concentration
pK_a	acidity constant
PNEC	predicted no effect concentration
PPCPs	pharmaceuticals and personal care products
QACs	quaternary ammonia compounds
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
sp.	species
t	ton
TU	toxic unit
WET	whole effluent toxicity
WHG	Wasserhaushaltsgesetz
WWT	wastewater treatment
WWTP	wastewater treatment plant

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Summary

Municipal wastewater contains nutrients valuable for a reuse in agriculture and can be the source of a multitude of chemicals used in private households and industry, too. As many of these chemicals are incompletely degraded during wastewater treatment, their residues remain partly in sewage sludge and partly in treated wastewater. Concerns are linked particularly to the so called micropollutants, i.e. anthropogenic organic substances such as personal care products, pharmaceuticals and biocides, for which scarce data on their degradability and environmental fate and particularly on their ecotoxicity are available. Thus, when reusing treated wastewater and sewage sludge for irrigation or as soil amendment for a sustainable land and water management, these wastewater-borne pollutants may enter soil, groundwater and surface water. The present work therefore aimed at assessing potential ecotoxic effects on aquatic and terrestrial organisms of reusing treated wastewater and sewage sludge. To this end, established as well as newly developed experimental approaches were used to investigate the problem on several levels. Individual wastewater-borne substances, samples from field study sites and samples from a soil column experiment simulating prolonged wastewater irrigation were examined.

At the start of the experimental work, the ecotoxicity of climbazole was characterised towards five aquatic and five terrestrial test organisms. Climbazole is an azole antimycotic agent applied in cosmetics and anti-dandruff shampoos and was recently detected in relatively high concentrations in treated wastewater and sewage sludge. In the present work climbazole was found to be particularly toxic towards plants such as water lentils with effective concentrations comparable to those of agricultural azole fungicides. Dwarfism, that is reduced shoot elongation observed in plants, pointed at a specific, phytohormone inhibiting mode of action of climbazole. Furthermore, the expected influence of the soil pH on the phytotoxicity of climbazole was experimentally confirmed.

Based on the findings for climbazole, two additional azole antimycotics, ketoconazole and fluconazole, and the regularly in sewage sludge detected biocide benzyldimethyldodecyl-ammonium chloride (BDDA) were investigated for their toxicity towards plants.

In aqueous medium, an increasing phytotoxicity from fluconazole to BDDA, ketoconazole and climbazole was observed, while in soil, phytotoxicity increased from BDDA to ketoconazole, climbazole and fluconazole. The relatively low terrestrial toxicity of BDDA and ketoconazole probably resulted from their strong binding to soil as well as their good biodegradability. To render the exposure scenario more realistic, sewage sludge was co-applied with the four test substances in a parallel test run. However, as no detectable influence on their effective concentrations was found, it can be assumed that the current practice of assessing sewage sludge borne substances with biotests in standard soil is sufficiently realistic.

In a further study, different advanced sludge-treatment technologies were assessed for their efficacy in reducing pollutants. Results from the present work indicated that effects assessed in terrestrial short term biotests only seldom correlated with the concentrations of certain pollutants. Rather, a negative correlation of the stability of the sludges, determined by the ratio of volatile to total solids, to their ecotoxicity was seen.

Another aspect of the present work was the design and performance of an experimental approach to assess the environmental risk of a long-term irrigation with treated wastewater concerning the quality of soil and water in a prospective way, i.e. before the installation at field scale. For the simulation of a continuous irrigation corresponding to approximately 30 years, a percolation apparatus was developed and four different soils were percolated with treated wastewater for three months. Acute and chronic biotests with nine test organisms from different trophic levels (green algae, water lentils and water fleas as well as oilseed rape, oats, bacteria, spring tails, enchytraeids and earthworms) were used to assess the soil percolates as well as the soils with and without percolation. These investigations were accompanied by a comprehensive chemical monitoring conducted by project partners. Results indicated that the soil passage, that is the percolation through the soil, generally improved the quality of the treated wastewater as habitat for aquatic organisms which was visible by a reduction of its phytotoxicity. However, in some cases it deteriorated the water quality, probably resulting from the leaching of metals from pre-contaminated soil. A deteriorated habitat quality of the soil after the percolation with treated wastewater was observed for several test organisms and soils. In the same, mainly peaty soils, the highest accumulation of wastewater-borne micropollutants and of zinc was measured. Yet, their concentrations did not correlate to the observed biological effects. Moreover, data on ecotoxicity were only available for a small fraction of the detected substances so that their concentrations could not successfully be used to predict expected biological effects.

The experimental approach used in the present work demonstrated to be an adequate tool to support the prospective evaluation of environmental risks of treated wastewater irrigation. Overall, it can be concluded that the reuse of treated wastewater on soil can improve the quality of treated wastewater but that this can come at the cost of deteriorating the quality of the soil. As these risks cannot be generalised, a comprehensive biotest battery as well as chemical analysis should be used to assess them on a case-specific basis for each respective wastewater and the respective soil.

Zusammenfassung

Wasser ist von fundamentaler Bedeutung für viele Bereiche des menschlichen Lebens, allen voran die Trinkwasserversorgung und die Lebensmittelproduktion. Angesichts steigender Wasserknappheit in großen Teilen der Welt, südeuropäischen Ländern wie Spanien und Griechenland, aber auch Regionen in Deutschland wie zum Beispiel Brandenburg, ist die Beregnung von landwirtschaftlichen Flächen ein Thema von zunehmender Bedeutung. Dazu tragen einerseits vermehrte Trockenperioden im Sommer bei, die eine Beregnung zur Sicherung des Ertrags auf immer mehr Flächen erforderlich machen. Gleichzeitig steigt der Verbrauch an Trinkwasser sowie an Brauchwasser für den häuslichen und industriellen Gebrauch in Städten und Ballungsräumen. Aus diesen Gründen ist der verantwortungsvolle Umgang mit der Ressource Wasser an der Schnittstelle von Land- und Wassermanagement eine herausfordernde Aufgabe.

Da Kläranlagen das gereinigte Abwasser in Fließgewässer oder Kanäle, sogenannte Vorfluter, einleiten, wird es aus dem Gebiet, dem es entzogen wurde, abtransportiert und geht damit der Region langfristig verloren. Auf der anderen Seite soll laut der EU-Wasserrahmenrichtlinie die Qualität von Gewässern einschließlich des Grundwassers langfristig verbessert werden, indem die Einträge von Nährstoffen und anthropogenen Chemikalien möglichst verringert werden. Eine Filtration durch Boden, auch Bodenpassage genannt, kann bekanntermaßen durch teilweisen Rückhalt oder Abbau von Nährstoffen und anderen Rückständen zur weitergehenden Reinigung des gereinigten Abwassers beitragen. Vor diesem Hintergrund scheint eine Wiederverwendung von gereinigtem Abwasser zu Beregnungszwecken sinnvoll, da sie eine landwirtschaftliche Produktion ermöglichen und den regionalen Wasserhaushalt stützen könnte, während sie gleichzeitig die Belastung von Oberflächengewässern reduzieren kann.

Klärschlamm, also das feste Endprodukt der Kläranlage, besteht zum größten Anteil aus organischem Kohlenstoff sowie Nährstoffen wie Stickstoff und Phosphor und kann daher einen wertvollen Dünger in der Landwirtschaft darstellen. Angesichts knapp werdender natürlicher Lagerstätten von Phosphor erscheint eine regionale Rückführung von durch Lebensmittelproduktion verbrauchten Nährstoffen in den Boden sinnvoll. Allerdings nimmt Klärschlamm auch sorptive und schwer abbaubare Substanzen wie organische Schadstoffe oder Schwermetalle aus dem Abwasser auf, die durch die Nutzung von Klärschlamm als Dünger in den Boden eingebracht werden.

Besondere Bedenken sowohl bei der Wiederverwendung von gereinigtem Abwasser als auch von Klärschlamm erregen dabei sogenannte *emerging pollutants* oder *micropollutants*, also anthropogene Substanzen aus Körperpflegeprodukten, Medikamenten und Bioziden, deren Abbaubarkeit und Verhalten in der Umwelt erst lückenhaft untersucht ist und für die in den meisten Fällen nur wenige ökotoxikologische Daten vorliegen.

Die vorliegende Dissertation hatte daher das Ziel, die Wiederverwendung von gereinigtem kommunalem Abwasser und Klärschlamm in der Landschaft hinsichtlich möglicher ökotoxikologischer Effekte auf aquatische und terrestrische Organismen zu bewerten. Um sich der Fragestellung auf verschiedenen Ebenen zu nähern, wurden sowohl etablierte experimentelle Methoden angewendet als auch neue Ansätze entwickelt. Untersucht wurde die Wirkung von im Abwasser anzutreffenden Einzelsubstanzen, von Umweltproben aus Feldstudien und von Proben aus einem Bodensäulenversuch zur Langzeitberegnung mit Abwasser.

Zunächst wurde die Ökotoxizität von Climbazol umfassend charakterisiert, welches kürzlich in relativen hohen Konzentrationen in Abwasser und Klärschlamm nachgewiesen wurde. Climbazol ist ein Antimykotikum aus der Wirkstoffgruppe der Azole und wird in Kosmetika als Konservierungsmittel und in Antischuppen-Shampoos eingesetzt. In Labortests nach internationalen Richtlinien wurden die Effekte der Substanz gegenüber fünf aquatischen und fünf terrestrischen Testorganismen untersucht. Es zeigte sich, dass Climbazol besonders gegenüber Primärproduzenten eine hohe Toxizität aufwies. Dabei lagen die abgeleiteten mittleren Effektkonzentrationen (EC_{50}) von Climbazol bei 11 mg/L gegenüber Wasserflöhen (*Daphnia magna*, Immobilität nach 48 h), 5.8 mg/L gegenüber Fischembryos (*Danio rerio*, Mortalität nach 48 h), 0.12 mg/L gegenüber Kieselalgen (*Navicula pelliculosa*, Inhibition des Biomassezuwachses nach 72 h), 0.16 mg/L gegenüber Grünalgen (*Pseudokirchneriella subcapitata,* Inhibition des Biomassezuwachses nach 72 h), und 0.01 mg/L gegenüber Wasserlinsen (*Lemna minor,* Inhibition des Biomassezuwachses nach 7 Tagen).

Climbazol schien keine Effekte auf Springschwänze (*Folsomia candida*, Reproduktion nach 28 Tagen) und Enchyträen (*Enchytraeus bigeminus*, Reproduktion nach 28 Tagen) bis zur höchsten Testkonzentration von 1000 mg/kg d.w. zu haben, während die terrestrische Toxizität gegenüber Bakterien (*Arthrobacter globiformis*, Dehydrogenase Aktivität nach 1 h) mit einer EC_{50} von 456 mg/kg d.w. gering und gegenüber den Pflanzen (Inhibition Sprossgewicht nach 14 Tagen) Hafer (*Avena sativa*) und Raps (*Brassica napus*) mit einer EC_{50} von 31 mg/kg d.w. und 19 mg/kg d.w. deutlich höher war.

Insgesamt lag die Toxizität von Climbazol in einem Konzentrationsbereich ähnlich dem landwirtschaftlich eingesetzter Azol-Fungizide. Der beobachtete Zwergwuchs in höheren Pflanzen wies außerdem auf die Inhibition bestimmter Phytohormone, Gibberelline und/oder Brassinosteroide hin. Dieser spezifische Wirkmechanismus ist auch für andere Azol-Fungizide bekannt und wird zum Teil gezielt zur Steuerung des Pflanzenwachstums eingesetzt.

Darüber hinaus wurde die Abhängigkeit der Climbazol-Toxizität vom pH-Wert des Bodens untersucht. Bei Climbazol handelt es sich um eine schwache Base, die im sauren pH-Bereich, also unterhalb seiner Säurekonstante (pK_a) von 7.5, zu mehr als 50% als Kation vorliegt. Gleichzeitig ist

bekannt, dass geladene Moleküle langsamer durch biologische Membranen aufgenommen werden als neutrale, ungeladene. Daher wurde vermutet, dass die Toxizität von Climbazol im sauren pH-Bereich geringer als im basischen pH-Bereich ist. Diese Hypothese wurde experimentell bestätigt, da bei einem basischen Boden-pH von 7.7 signifikant höhere Effekte in *B. napus* als bei einem sauren pH-Bereich von pH 5.5 beobachtet wurden. Der parallel untersuchte Einfluss des organischen Kohlenstoffgehalts (C_{org}) des Bodens erwies sich dagegen als weniger bedeutsam in Bezug auf die Toxizität von Climbazol. Lediglich die Inhibition der Keimrate war bei dem Bodengehalt von 5% C_{org} signifikant höher als bei dem Bodengehalt von 10% C_{org}, während keine Unterschiede in der Inhibition der Sprosslänge und des Frischgewichts festgestellt wurden. Angesichts der relativ hohen Hydrophobie von Climbazol mit einem Oktanol-Wasser-Verteilungskoeffizienten (log K_{ow}) von 3.33 war dieses Ergebnis überraschend, da bei einem hohen C_{org} Gehalt eine starke Sorption der Testsubstanz im Boden vermutet worden war. Allerdings können Pflanzen, im Gegensatz zu anderen Bodenlebewesen, die Aufnahme von Substanzen u.a. über eine Änderung des Boden-pH im Rhizosphärenbereich aktiv beeinflussen. Auf diese Weise können auch sorbierte oder geladene Substanzen für Pflanzen verfügbar sein, was den geringen Einfluss des C_{org}-Gehalts des Bodens auf die Phytotoxizität von Climbazol erklären könnte.

Basierend auf den Erkenntnissen zu Climbazol wurden im zweiten Schwerpunkt der vorliegenden Arbeit zwei weitere Azol-Antimykotika, Ketoconazol und Fluconazol, sowie das regelmäßig in Klärschlamm vorzufindende Biozid Benzyldimethyldodecylammoniumchlorid (BDDA) auf ihre Toxizität gegenüber den Primärproduzenten *L. minor* und *B. napus* hin untersucht. Ketoconazol und Fluconazol werden in Antischuppen-Shampoos beziehungsweise in Cremes als Medikament gegen Hautpilz eingesetzt, während BDDA als kationisches Tensid vielseitige Verwendung unter anderem in Desinfektionsmitteln, als Korrosionsschutz in Spülmitteln oder als Biozid gegen Algenwuchs in Schwimmbädern findet. Neben ihrer unterschiedlichen Anwendung unterschieden sich die Substanzen auch in ihren physikalisch-chemischen Eigenschaften und dem damit verbundenen Umweltverhalten. Aufgrund der Hydrophobie (log K_{ow} von 4.3) von Ketoconazol und der Hydrophilie von Fluconazol (log K_{ow} von 0.5) sorbiert das ungeladene Molekül von Ersterem stark und Letzterem schwach. Basierend auf ihren pK_a Werten steigt der kationische Anteil von Ketoconazol ähnlich wie bei Climbazol mit abnehmendem pH-Wert des Mediums, während Fluconazol im umweltrelevanten Bereich zwischen pH 3 und 10 ausschließlich als ungeladenes Molekül vorliegt. Dagegen liegt BDDA in Wasser permanent als Kation vor, ist also sowohl gut wasserlöslich als auch stark an negative Oberflächen wie Klärschlamm oder Boden sorbierend.

Alle drei Substanzen erwiesen sich als äußerst toxisch gegenüber *L. minor* mit EC_{50}-Werten von 0.97 mg/L, 0.30 mg/L bzw. 0.06 mg/L für Fluconazol, BDDA bzw. Ketoconazol (bezogen Inhibition des Frondzahlzuwachses und gemessene Konzentrationen). Damit ist deren Toxizität nur wenig geringer als die von Climbazol. Chemische Analytik der Testlösungen zu Beginn und Ende der Biotests

zeigten überdies eine geringe Wiederfindung der nominalen Konzentrationen von BDDA (12%) und Ketoconazol (44%), was auf Sorption an Glasgefäße und Wasserlinsen aber vermutlich großenteils auf Abbau zurückzuführen ist.

Gegenüber *B. napus* zeigten Ketoconazol und BDDA dagegen eine deutlich geringere Toxizität als Climbazol und Fluconazol mit EC_{50}-Werten von 578 mg/kg d.w., 222 mg/kg d.w., 11 mg/kg d.w. bzw. 9.2 mg/kg d.w. (bezogen auf Inhibition des Frischgewichts). Die geringe terrestrische Toxizität von BDDA und Ketoconazol hängt vermutlich mit ihrer starken Bindung an den Boden sowie ihrer relativ guten Abbaubarkeit zusammen. Anders als bei den aquatischen Tests wurden die Testkonzentrationen jedoch nicht analytisch verifiziert, so dass davon auszugehen ist, dass die auf nominalen Bodenkonzentrationen basierenden EC_{50}-Werte die tatsächliche Toxizität von zumindest BDDA und Ketoconazol unterschätzen.

Vergleicht man die ermittelte Toxizität mit berichteten Konzentrationen in der Umwelt, so scheint aktuell keine der vier Substanzen eine direkte Gefahr für die Umwelt darzustellen. Allerdings liegen die gemessenen Konzentrationen von BDDA und Climbazol in Klärschlamm und gereinigtem Abwasser nur maximal um den Faktor 15 über den Effektkonzentrationen für Pflanzen, so dass die Sicherheitsabstände für diese Substanzen relativ gering sind, und ihr Vorkommen in der Umwelt weiterhin kritisch verfolgt werden sollte.

Anhand der drei Azole und BDDA sollte überdies der Einfluss von Klärschlamm als relevante Expositionsmatrix für abwasserbürtige Spurenstoffe gegenüber Pflanzen untersucht werden. Dazu wurde die Toxizität der Substanzen parallel zu den Biotests in Standardboden in einem mit Klärschlamm versetzten Standardboden getestet. Die eingesetzte Menge an Klärschlamm von 3.8 g/kg d.w. Boden entsprach dabei der in Deutschland maximal erlaubten Rate von 5 t/ha und 10 cm Einarbeitungstiefe. Es zeigte sich, dass die gleichzeitige Zugabe von Klärschlamm zu den vier applizierten Testsubstanzen keinen statistisch feststellbaren Einfluss auf deren EC_{50} gegenüber *B. napus* hatte. Basierend auf diesen Ergebnissen kann davon ausgegangen werden, dass die aktuelle Testung von klärschlammbürtigen Substanzen über Biotests in Standardboden ausreichend realistisch ist.

In einer dritten Fragestellung der vorliegenden Arbeit sollte die Effektivität verschiedener Techniken zur weitergehenden Behandlung von Klärschlamm zur Reduktion von Schadstoffen untersucht werden. Dazu wurden von Projektpartnern drei Techniken an Pilot- oder Laboranlagen angewendet: i) thermophile anaerobe Stabilisierung mit und ohne vorherige thermale Hydrolyse, ii) zweistufige mesophile/thermophile anaerobe Stabilisierung mit und ohne vorherige Ultraschallbehandlung und iii) sequentielle anaerobe/aerobe Stabilisierung. In der vorliegenden Arbeit wurden die Schlammproben aus verschiedenen Abschnitten der Prozesse mittels dem Bakterienkontakttest (*A. globiformis*) und dem Regenwurmfluchttest (*Eisenia fetida*) untersucht. Diese Biotests wurden

ausgewählt, weil sie sich in der Testung von Abfallproben als geeignet erwiesen haben, mit Destruenten für wichtige Bodenorganismen repräsentativ sind, nur kurze Zeit dauern (1 h, bzw. 2 Tage) und geringe Probenvolumina erfordern. In den Biotests konnten Unterschiede zwischen der Habitatqualität der Proben ermittelt werden, wobei die Stabilisierung meist zu einer Verringerung der Effekte im Vergleich zum unbehandelten Schlamm führte. Alle stabilisierten Klärschlämme zeigten negative Effekte erst ab Konzentrationen, welche um mindestens Faktor 30 über den anzunehmenden Bodenkonzentrationen bei üblichen Applikationsraten lagen (0.8 g/kg d.w., ausgehend von 2 t/ha und 20 cm Einarbeitungstiefe). Darüber hinaus wurde der Einfluss der von Projektpartnern analysierten Schadstoffe überprüft. Ein Zusammenhang zwischen den beobachteten Effekten und den Substanzkonzentrationen konnte allerdings nur für Naphtalen, Triclocarban und Carbamazepin statistisch nachgewiesen werden. Vielmehr bestand eine deutliche negative Korrelation zwischen der Toxizität gegenüber *A. globiformis* und der Schlammstabilität, gemessen am Verhältnis zwischen organischem und gesamtem Feststoffgehalt. Die Ergebnisse weisen somit darauf hin, dass eine Reduzierung der Ökotoxizität des Klärschlamms besser durch eine gute Stabilisierung des Klärschlamms und eine damit verbundene geringere Freisetzung von sorbierten Schadstoffen zu erreichen ist, als durch einen optimierten Abbau, welcher zu einer minimalen Reduzierung der absoluten Schadstoffkonzentrationen im Klärschlamm führt.

Der vierte Fokus der vorliegenden Arbeit lag auf der Entwicklung und Durchführung eines experimentellen Ansatzes um das Umweltrisiko einer Langzeitberegnung mit gereinigtem Abwasser prospektiv abschätzen zu können. Mittels einer im Rahmen dieser Untersuchung entwickelten Durchflussapparatur wurden vier Böden für drei Monate mit einem Abwasservolumen beaufschlagt, das ungefähr einer kontinuierlicher Verregnung über einen Zeitraum von 30 Jahren entsprach. Drei der Böden entstammten dem alten Rieselfeld Hobrechtsfelde am Rande Berlins, einer Modellfläche des Projektes ELaN, wobei ein Boden stark („s1“), ein weiter gering („s2“) und der dritte („s3“) nicht vorbelastet war. Als vierter Boden wurde ein Standardboden („s4“) verwendet.

Akute und chronische Biotests wurden angewendet um das Abwasser vor und nach der Passage durch die Bodensäulen sowie die Böden mit und ohne dreimonatige Abwasserbeaufschlagung zu untersuchen. Als aquatische Testorganismen dienten Grünalgen (*P. subcapitata*), Wasserlinsen (*L. minor*) und Wasserflöhe (*D. magna*). Als terrestrische Testorganismen wurden Springschwänze (*F. candida*), Enchyträen (*E. crypticus*), Regenwürmer (*E. fetida*), Bakterien (*A. globiformis*), Hafer (*A. sativa*) und Raps (*B. napus*) eingesetzt. Diese Untersuchungen wurden durch Projektpartner mit einem chemisch-analytischen Messprogramm begleitet.

Es zeigte sich, dass das gereinigte Abwasser eine deutliche Phytotoxizität gegenüber *P. subcapitata* und *L. minor* aufwies, die durch die Bodenpassage, vor allem durch die torfigen Böden s2 und s3, in der Regel abnahm. Hier konnte demnach die Qualität des gereinigten Abwassers als Habitat für

aquatische Organismen durch die Bodenpassage verbessert werden. Allerdings wurde in einigen Sickerwässern auch eine Zunahme der Toxizität gegenüber *P. subcapitata* beobachtet, was wahrscheinlich auf die Auswaschung von Metallen aus dem vorbelasteten Boden zurückzuführen ist. Die Qualität des Bodens als Habitat für terrestrische Organismen wurde durch die Abwasserbeaufschlagung für einige Testorganismen signifikant verschlechtert: für *E. crypticus* in s1, für *A. globiformis* in s2 und für *A. sativa* in s3, verglichen zum jeweiligen Boden ohne Beaufschlagung. Vor allem in den torfigen Böden s2 und s3 wurde die höchste Anreicherung von abwasserbürtigen Spurenstoffen sowie von Zink gemessen, was mit ihrer erhöhten Reinigungsleistung bezogen auf das Sickerwasser übereinstimmte. Allerdings konnte keine direkte Korrelation zwischen bestimmten Substanzen und den biologischen Effekten festgestellt werden. Auch eine theoretische Abschätzung der Effekte basierend auf den gemessenen Substanzkonzentrationen über den *Toxic Units* Ansatz erwies sich als nicht erfolgreich, weil ökotoxikologische Daten nur zu einem geringen Teil der analysierten Substanzen vorlagen. Insgesamt erwies der Versuch damit, dass die Verregnung von gereinigtem Abwasser zwar die Qualität des Abwassers verbessern kann, dies aber mit einer Beeinträchtigung der Bodenqualität einhergehen kann. Eine prospektive Risikobeurteilung von Abwasserverrieselung sollte daher fallspezifisch für das zu verwendende Abwasser und den entsprechenden Boden durchgeführt werden. Zur Beurteilung der Habitatqualität eignet sich eine Biotestbatterie, die unterschiedliche trophische Ebenen abdeckt, und bestenfalls durch *in vitro* Tests zum Feststellen spezifischer, sublethaler Effekte erweitert wird. Chemische Analytik ergänzt das Bild um das Verhalten von Substanzen, deren Konzentrationen nicht unmittelbar Effekte zeigen, und kann zum besseren Verständnis möglicher Ursachen für die beobachteten biologischen Effekte dienen. Eine kausale Verknüpfung von Ökotoxizität und Schadstoffen ist jedoch bei Umweltproben mit einer derart komplexen Zusammensetzung wie Abwasser und Boden kaum eindeutig möglich, so dass biologische und chemische Ergebnisse als sich gegenseitig ergänzende Informationen betrachtet werden sollten.

1. General introduction

In the following chapter, the theoretical background and the ensuing objectives of the present thesis are stated. To start with, the scope of the thesis in the context of two research projects is introduced. Subsequently, the controversial discussion on the topic is summarised, complemented by the regulatory context and assessment approaches. Finally, the current state of research and the specific research objectives and hypothesis of the present thesis are provided.

1.1. Scope of the present thesis

The overall aim of the present thesis was to assess and evaluate the ecotoxicological hazard of using treated wastewater for irrigation of soil and of sewage sludge as soil amendment. The work was financially supported by two independent projects which complemented each other well as they focussed on different products of municipal wastewater treatment:

Questions related to treated wastewater were addressed within the project ELaN ("Entwicklung eines integrierten Landmanagements durch nachhaltige Wasser- und Stoffnutzung in Nordostdeutschland", supported by the Federal Ministry of Education and Research, FKZ: 033L025F, duration: 1/2011 – 12/2014), which aimed at developing an integrated land management through sustainable water and nutrient reuse in north-eastern Germany. The use of treated wastewater for cultivating energy crops and supporting the regional water balance, which in Germany is legally not provided for, was tested in an urban model site close to Berlin and a rural model site in Brandenburg. In particular, the present work intended to determine the environmental impacts in order to support the prospective risk evaluation and management process. Besides the results discussed in the present thesis and in the publications provided in annexes A.1.1 and A.1.4, field samples from the two model sites were assessed, firstly with water samples taken along the flow path of treated wastewater in the field, and secondly with soil before and after three summers of irrigation with treated wastewater. These results are available in the final project report (http://www.elan-bb.de) and will be published in an international journal (Balla et al., in preparation).

Sewage sludge related questions were addressed in the project ROUTES ("novel processing routes for effective sewage sludge management", supported by the European Union, Contract No 265156, FP7 2007-2013, THEME [ENV.2010.3.1.1-2], duration: 5/2011 – 4/2014) which intended to develop innovative system solutions for municipal sludge treatment and management. Advanced sludge treatment technologies were tested for their efficiency in degrading wastewater-borne pollutants and stabilising the final sludge. In particular, the present work intended at determining the quality of the differently treated sludges and broadening the knowledge on the ecotoxicity of sludge-associated

contaminants. Besides the results discussed in the present thesis and in annex A.1.2 and A.1.3, findings from additional experiments with sewage sludge samples are available in respective project reports (http://www.eu-routes.org) and in a congress book chapter (Coors et al., 2013).

1.2. Motives and concerns in reusing treated wastewater and sewage sludge

Water is of fundamental importance for maintaining landscape functions, for food production as well as for drinking water supply. Irregular precipitation, draughts and intensified agriculture (Botti et al., 2009, Bedbabis et al., 2014) drive the need for irrigation in horticulture, crop production (Hamilton et al., 2007, Angelakis et al., 1999) and water demanding plants and energy crops (Dorta-Santos et al., 2014). In contrast, increasing drinking and domestic water consumption render groundwater too valuable for irrigation (Ilias et al., 2014). Particularly in arid zones, this trend makes a resource saving water management indispensable. Meanwhile, wastewater treatment plants (WWTP) especially in urban areas discharge high and relatively constant volumes of treated wastewater into surface waters (Fatta-Kassinos et al., 2011, Eggen et al., 2014). This discharge route ensures instant dilution of nutrients and wastewater-borne pollutants and thus minimises their risk to the environment.

Treated wastewater possesses several characteristics that render it a potentially valuable resource for soil and landscape management (Hamilton et al., 2007, Muyen et al., 2011, Dorta-Santos et al., 2014): Being continuously produced at WWTPs it can be an easily available water supply for nearby agriculture (Ternes et al., 2007, Graber and Gerstl, 2011, Ilias et al., 2014). Low productivity or abandoned land can be used cost-effectively for cultivation of energy crops such as willows or establishment of public green areas by applying treated wastewater for fertirrigation, i.e. fertilise and irrigate with one medium (Teijon et al., 2010, Dorta-Santos et al., 2014).

An alternative application of treated wastewater is the management of wetlands or degraded fens (Hamilton et al., 2007). Preventing these landscapes from falling dry serves the natural storage of carbon dioxide and the conservation of a versatile landscape (Lischeid and Nathkin, 2011).

During wastewater treatment, removal of pollutants is often incomplete. Some of the remaining pollutants are present at very low concentrations and are therefore called micropollutants. The conventional discharge of treated wastewater into surface waters releases micropollutants which may be harmful to the aquatic environment. In contrast, soil passage, i.e. the percolation through the soil body, can retain sorptive pollutants in the soil matrix and enhance pollutant removal by increasing the reaction surface for microbial degradation and encompassing aerobic as well as anaerobic conditions (Ternes et al., 2007, Scheurer et al., 2015). This process is made use of in constructed wetlands which are applied solely or as further purification step (Haberl, 2003, Cordy et al., 2004, Verlicchi and Zambello, 2014).

Globally, an estimated area of approximately 20 million ha is irrigated with raw or treated wastewater today (Hamilton et al., 2007). The use of treated wastewater for agricultural irrigation purposes is particularly important in arid regions like Australia, Israel or California (Muyen et al., 2011, Hamilton et al., 2007). However, treated wastewater as additional source for landscape management has gained recent interest on the European Union (EU) level and triggered several research initiatives (COM, 2012, JRC, 2014). Particularly southern European countries like Spain, Italy and Greece examine the potential of reusing treated wastewater in different application schemes (Angelakis et al., 1999, Botti et al., 2009, Dorta-Santos et al., 2014, Ilias et al., 2014). In view of the increasing urgency of this issue, the European Commission aims at proposing a suitable EU-level regulation on common environmental and health standards for reused water by the year 2015 (COM, 2012, JRC, 2014).

In addition to wastewater, digested sewage sludge, the solid remains of wastewater treatment (WWT), is potentially valuable for land management (Fytili and Zabaniotou, 2008, Muyen et al., 2011, Schowanek et al., 2004, Tyagi and Lo, 2013). Additionally, the costs of artificial fertilisers increase and are expected to rise particularly for phosphorus as this essential nutrient has limited natural storages in only few countries (Tyagi and Lo, 2013). Sewage sludge represents an inexpensive and widely available source of nutrients, primarily nitrogen, phosphorus, potassium, sulphur and magnesium but also organic matter important to improve drainage and water holding capacity of soils (Fytili and Zabaniotou, 2008). In Germany, sewage sludge of about 1.9 million t dry weight was produced in 2010, of which 53% was incinerated, 30% was used in agriculture and 17% for landscaping and land reclamation (Statistisches Bundesamt, 2013).

Especially in regions where dung and manure from animals is not sufficient to meet the demand for organic fertiliser, sewage sludge can be a valuable and budget alternative (Petersen et al., 2003, Mousavi et al., 2013). In the USA and Canada, sewage sludge that meets certain criteria for use in agriculture, mainly related to pathogens and heavy metals, is named biosolids and its use as soil amendment is common practice (Topp et al., 2008). From an economic point of view, sewage sludge disposal to agricultural land is attractive for wastewater treatment plants as it is less cost intensive than incineration (Schowanek et al., 2004). Potential benefits of the reuse of treated wastewater for irrigating soil and improving regional water management and of sewage sludge for amending soil possesses are summarised at the end of this chapter (see Table 1).

Notwithstanding the benefits of using treated wastewater and/or sewage sludge for irrigation and/or soil amendment, this practice meets increasing concerns, partly due to well-known problems, partly due to unknown risks. Long-term application of treated wastewater as practiced in arid regions like Israel, California and great parts of Australia can lead to salt accumulation causing plant growth inhibition and negative effects on soil structure (Muyen et al., 2011, Graber and Gerstl, 2011). On

soils where the passage time from surface to groundwater is particularly short, use of treated wastewater or sewage sludge can cause the breakthrough of salts into groundwater and thereby threaten drinking water production (Cordy et al., 2004, Postigo and Barcelo, 2015).

Of increasing concern are hygienic, toxicological and ecotoxicological hazards related to the use of treated wastewater and sewage sludge on land (Angelakis et al., 1999, Malchi et al., 2014). While the contamination with heavy metals has been well studied and is regulated in most countries nowadays (Mousavi et al., 2013), worries mainly relate to the great number of micropollutants such as pharmaceuticals and personal care products (PPCPs) and their persistent break down products, whose environmental effects and risks to human health are not well understood yet (Daughton and Ternes 1999, Muyen et al., 2011, Clarke and Smith, 2011, Eggen et al., 2014). In the following, the occurrence and potential hazards of wastewater-borne pollutants are shortly addressed.

Pollution of wastewater and sewage sludge

WWTPs are designed to remove major nutrients by turning nitrogen into bacterial biomass and by precipitating phosphorus from the water phase to counteract eutrophication of receiving waters (Ternes et al., 2004, Eggen et al., 2014). Municipal wastewater generally consists of a mixture of domestic and industrial wastewater and in case of so called mixed sewers also of stormwater, i.e. precipitation discharges (Eriksson et al., 2008). In Germany, 95% of the wastewater is treated in municipal WWTPs and mostly (>93%) passes tertiary treatment in the WWTP, i.e. a mechanical filtering (sand filter and sedimentation), nitrogen removal (a denitrification and a nitrification step) and phosphorous removal (precipitation, either biologically or by the addition of e.g. iron) (Ternes et al., 2004, UBA, 2013). The removal of micropollutants such as PPCPs during wastewater treatment (WWT) is thus a side-effect and can range from no to complete degradation depending on each substance (Ternes, 1998, Gros et al., 2010, Kosma et al., 2014). In the EU, about 100 000 different chemicals are registered (COM, 2001), of which many at some stage enter wastewater so that the contamination spectrum of raw and treated wastewater is potentially very large and diverse (Peysson and Vulliet, 2013, Eggen et al., 2014). Additionally, hospital effluents usually reach municipal sewer networks without preliminary treatment and thus represent an important immission source of a variety of pharmaceutically active compounds (Perrodin et al., 2013).

Depending on their physic-chemical properties, residues and transformation products of contaminants partition into the solid phase, i.e. sewage sludge, or stay in the aqueous phase, i.e. effluent (Ternes et al., 2004).

Treated wastewater can contain a multitude of hydrophilic, i.e. polar, ionised and water-soluble substances but usually in the range of µg/L to ng/L (Eggen et al., 2014). This poses challenges in view of their identification (what substances shall be monitored), quantification (need for analytical

methods with low detection limits) and characterisation (degradability, ecotoxicological effects) (Munoz et al., 2008, Teijon et al., 2010, von der Ohe, 2011). Pollutant residues released into the aquatic environment tend to persist in the water body, if they are not easily photochemically degradable in surface waters (Eggen et al., 2014). Even if micropollutants may be degraded eventually, they can be regarded as "pseudopersistent compounds" because of their continuous introduction in the environment (Hernando et al., 2006). Representative substances from different applications that are typically detected in treated municipal wastewater are depicted in Figure 1.

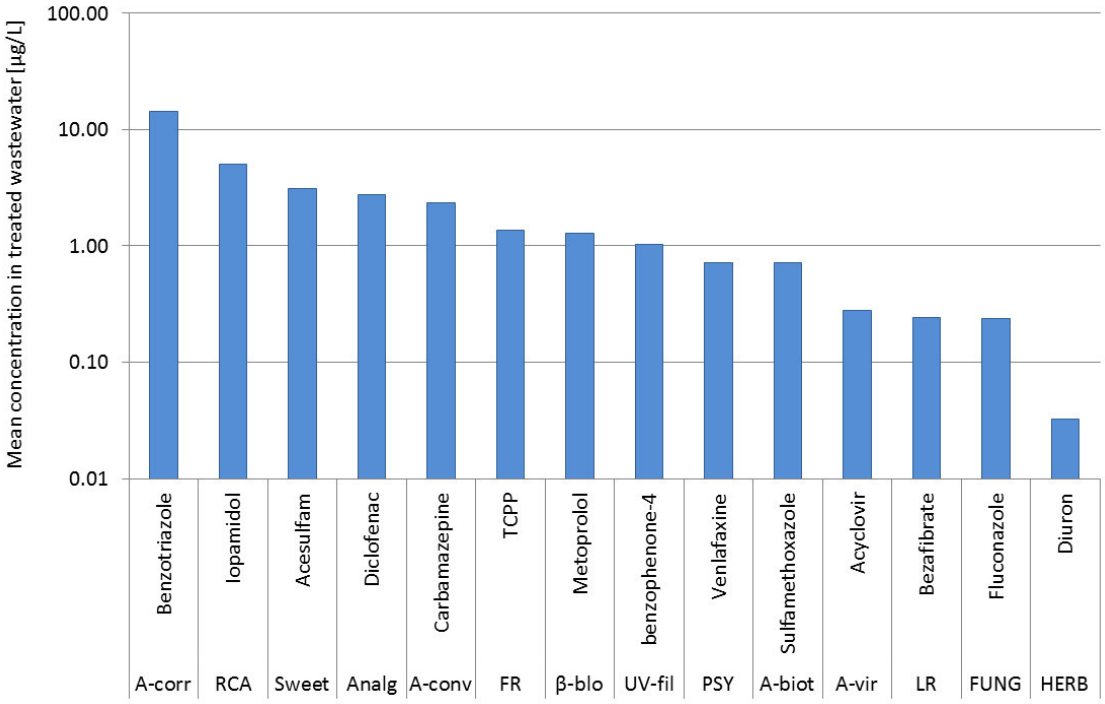


Figure 1: Representative organic micropollutants from different applications and their mean concentrations in treated municipal wastewater measured at a Berlin WWTP from September to December 2012 (data available in Richter et al., 2015, Annex 1.4). A-corr = anticorrosive agent, RCA = radiocontrast agent, Sweet = artificial sweetener, Analg = analgesic agent, A-conv = anticonvulsant, FR = flame retardant, β-blo = beta blocker, UV-fil = UV-filter, PSY = psycho-active drug, A-biot = antibiotic agent, A-vir = antiviral agent, LR = lipid regulator, FUNG = fungicide, HERB = herbicide.

Sewage sludge represents a sink particularly for sorptive substances with lipophilic character or positive charge due to its high content of organic matter (45% - 90%), which is a mixture of fats, proteins, carbohydrates (sugars, celluloses), lignin, amino acids, humic material, and fatty acids (Rogers, 1996, Clarke and Smith, 2011, Schowanek et al, 2004). The presence of heavy metals and persistent organic pollutants such as PAHs, PCBs, and EOX in sewage sludge at high concentrations (mg/kg) is well known (Eriksson et al., 2008, Mousavi et al., 2013). Besides these "classical pollutants", an unknown number of organic micropollutants is retained in sewage sludge (Eriksson et al., 2008, Ternes et al., 2004, Clarke and Smith, 2011) e.g. of surfactants such as linear alkyl sulfonates (LASs) and quaternary ammonia compounds (QACs) (see Figure 2). The occurrence of

these emerging pollutants in sewage sludge is less well investigated than for effluent, due primarily to a lack of appropriate methodologies (Peyson and Vulliet, 2013). The challenge of analysing the chemical composition of sewage sludge lies in the complex solid matrix which obstructs the chemical measurement, necessitating elaborate and time-consuming sample preparation steps before the identification, quantification and characterisation itself (Rogers, 1996, Barron et al., 2008, Peyson and Vulliet, 2013).

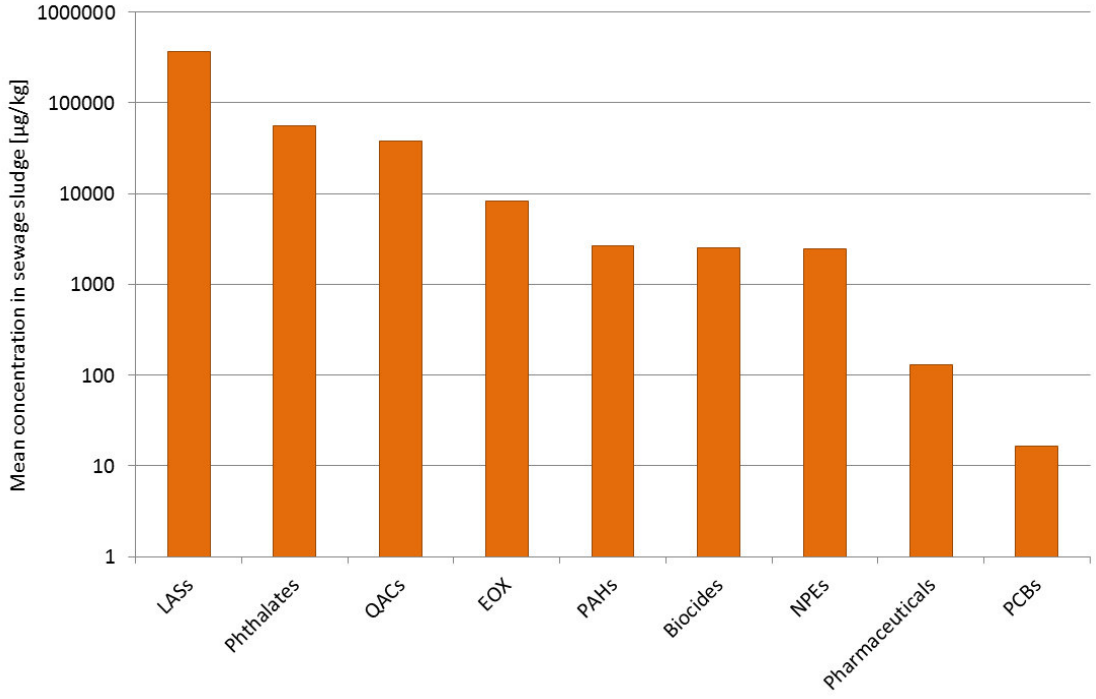


Figure 2: Representative organic pollutants in municipal secondary sewage sludge and their mean concentrations measured at a Rome wastewater treatment plant, April 2012 (data available in Braguglia et al., 2014, Annex 1.3). LASs = linear alkyl sulfonates, QACs = quaternary ammonia compounds, EOX = extractable organic halogens, PAHs = polyaromatic hydrocarbons, NPEs = nonylphenol ethoxylates, PCBs = polychlorinated biphenyls.

Prior to its application on land, sewage sludge is typically treated by a combination of thickening, anaerobic digestion, composting, lime stabilization, dewatering and/or thermal drying (Schowanek et al., 2004, UBA 2013). This treatment aims at minimising odour potential, avoiding microbial health risks, and minimising sludge volume (Schowanek et al., 2004, Natal-da-Luz et al., 2011) but may also concentrate present pollutants and increase binding of micropollutants to sewage sludge and thus hinder further microbial degradation.

Using sewage sludge as soil amendment leads to its dilution with soil; yet, heavy metals and other persistent organic pollutants may be retained in the soil and thus accumulate over time (Mousavi et al., 2013). Even for chemicals that degrade readily, if present in high concentrations and applied repeatedly, the soil concentrations may be significantly elevated (Harrison et al., 2006).

Ecotoxicological hazard of wastewater-borne pollutants

The concentrations of active pharmaceutical ingredients measured in the environment are mostly significantly below the therapeutic doses necessary for human treatment. However, the sensitivity of non-target organisms in the environment to the comparatively low but permanent exposure to a mixture of pharmaceutical residues is still unknown for the majority of substances and their transformation products (Daughton and Ternes, 1999, Boxall et al., 2012). For several organic micropollutants, aquatic organisms are sensitive at very low concentrations. For example, the analgesic agent diclofenac, the anticonvulsant carbamazepine and the beta-blocker metoprolol were found to exert cytotoxicity in fish (*Oncorhynchus mykiss* and *Cyprinus carpio*) at environmentally relevant concentration of 1 µg/L (Triebskorn et al., 2007). Similarly, the antidepressant fluoxetine is toxic to green algae (*Desmodesmus subspicatus)* at concentrations close to those found in the environment (Oaks et al., 2010). Another group of great environmental relevance are antibiotics, due to their high biological activity but also in view of promoting selection of antibiotic resistant bacterial strains (Kools et al., 2008, Boxall et al., 2012). Besides pharmaceuticals, biocides may be hazardous to the environment as they are designed to eliminate living organisms such as bacteria or algae. The anti-fouling agents isoproturon, diuron and terbutryn prevent the growth of biofilms on wood and facades. When leached during rain into sewers or surface waters they constitute a severe risk to the aquatic environment (Quednow and Püttmann, 2007, Köck-Schulmeyer et al., 2013).

Of recent concern is the risk from endocrine disruptors, i.e. substances which can affect the endocrine system. Estrogenically active compounds like androgen and estrogen hormones, e.g. estradiol and ethinylestradiol, are well removed by biological WWT mostly due to their lipophilicity related sorption to sludge. Thus, with few exceptions such as estrone, hormones are detected in treated wastewater at very low concentrations (e.g. lower than 7 ng/L, Daughton and Ternes, 1999, Joss et al., 2005, Leusch et al., 2014). Still, concentrations in wastewater receiving water bodies may affect wildlife, particularly fish (Henneberg et al., 2014). The artificial steroid 17α-ethinyl estradiole was shown to affect fish (*Oncorhynchus mykiss,* indicated by the estrogenic biomarker vitellogenin) at median effective concentration (EC_{50}) between 0.95 and 1.8 ng L/1 (Thorpe et al., 2003). The plasticiser bisphenol A exerts estrogen activity at very low concentrations as e.g. observed in the formation of super females in freshwater snails (*Marisa cornuarietis*) and marine prosobranchs (*Nucella lapillus*) at 1 µg/L upwards (Oehlmann et al., 2000). Considering these findings on selected micropollutants, it is no surprise that treated wastewater often exerts direct ecotoxic effects in *in vitro* and *in vivo* biotests (Leusch et al., 2014).

The spread of sewage sludge on agricultural soils has been controversially discussed for some decades mainly due to uncertainty about the risks associated with emerging pollutants transferred to soil (Peyson and Vulliet, 2013). In addition, leaching of sludge-associated pollutants to groundwater

and adjacent surface water can occur, mostly in case of water-soluble compounds and metabolites, but also via particle bound transport of sorptive substances (Schowanek et al., 2004, Topp et al., 2008).

Thus, sewage sludge application to soil may have negative impacts on soil microflora, terrestrial and aquatic organisms (Schowanek et al., 2004). Yet, the ecotoxicity of sewage sludge associated micropollutants toward soil fauna and flora has not been well studied so far (Natal-da-Luz et al., 2009, Clarke and Smith, 2011, Roig et al., 2012). In the attempt to overcome this lack, aquatic data are sometimes used to roughly estimate effects to terrestrial organisms (Munoz et al., 2009).

In summary, the application of treated wastewater and sewage sludge to land can potentially bear several hazards besides the before mentioned benefits (Table 1):

Table 1: Beneficial and hazardous characteristics of treated wastewater and sewage sludge linked to their application in land management.

Product	Beneficial characteristics	Hazardous characteristics
Treated wastewater	water resource for irrigation	salt accumulation in soil
	nutrient resource for easily accessible nutrients	leaching of nutrients and wastewater-borne pollutants to groundwater
	continuously available as by-product from wastewater treatment	retention in soil of wastewater-borne contaminants (mainly lipophilic and positively charged substances)
	groundwater recharge and regional water balance support	uptake of pollutants into crops for animal or human consumption
	soil passage further reduces nutrient and pollutant input to surface waters	transfer of pathogens to crops for animal or human consumption
		negative effects of pollutants to terrestrial and aquatic organisms
Sewage sludge	rich in organic carbon	negative effects on soil from high ammonia levels
	rich in easily accessible nutrients	leaching of nutrient surplus and sludge-borne pollutants to surface and groundwater
	continuously available as by-product from wastewater treatment	accumulation in soil of sludge-borne contaminants (mainly heavy metals and lipophilic organic pollutants)
	easy and cost efficient way of disposal	uptake of pollutants into crops for animal or human consumption
		transfer of pathogens to crops for animal or human consumption
		negative effects of pollutants to terrestrial and aquatic environment

1.3. Regulatory context

While on the EU and German level, the use of sewage sludge as fertiliser and soil amendment is regulated, the use of treated wastewater in land management is not foreseen so that no respective regulation exists (JRC, 2014). Yet, there are several environmental Directives that should be taken

into account when considering other applications of treated wastewater than its common discharge into surface waters.

Disposal of treated wastewater and sewage sludge in the EU and Germany

On the European level, the quality of surface waters and groundwater is regulated by the European Water Framework Directive (WFD, 2000/60/EC), outlining measures to achieve a good hygienic, ecological and chemical status of water bodies. The directive envisages monitoring of currently 45 priority substances (2000/60/EC, annex X; Directive on Environmental Quality Standards 2008/105/EC, amended by Directive 2013/39/EU) including several heavy metals, industrial chemicals such as PAHs and PCBs, and some pesticides and biocides such as DDT, diuron and terbutryn. Environmental quality standards (EQS) for these substances define threshold concentrations that are considered safe for aquatic organisms and shall not be exceeded in surface waters. Further substances from the group of pharmaceuticals may be considered in the future, e.g. diclofenac, 17α-ethinylestradiol and 17β-estradiol (2013/39/EU).

In Germany, wastewater has to be treated according to the current technical status before it is discharged into surface waters as stipulated since 1976 by the Federal Water Act (§ 57 WHG). Treated wastewater must not exceed specified nitrogen and phosphorous concentrations and the sum parameters chemical and biochemical oxygen demand. For the effluent discharge of certain industrial branches such as paper mills, chemical or food processing industry, the performance of bioassays on green algae, daphnia, fish and/or luminescent bacteria are requested by the Wastewater Ordinance (AbwV).

German legislation on groundwater follows the “precautionary principle”, i.e. any deterioration of the quantity and chemical quality of groundwater is to be avoided (WHG §47). Threshold values are defined for some compounds, e.g. for heavy metals, nitrate, chloride, and sulphate, and for sum concentrations of biocides and pesticides (GrwV). The immission of other substances is only allowed if a negative effect on groundwater quality can be excluded (GrwV). The use of treated wastewater for irrigation and landscape management is currently not foreseen in Germany but is exceptionally practised in some cases with approval of the local water authority (Ternes et al., 2007, Lottermoser et al., 2012).

The use of sludge in agriculture is encouraged by the current European sewage sludge directive as long as soil, vegetation, animals and humans are not threatened (86/278/EEC). Sludge should be applied via incorporation into soil, with restriction regarding the use in fruit and vegetable crops and on pastures. The directive requires that sludge is sufficiently treated, i.e. stabilised by biological, chemical or heat treatment or by long-term storage, and meets specified threshold values for heavy metals. According to German law, sewage sludge can be applied as fertiliser at a maximum volume

of 5 t dry weight per ha in three years (AbfKlärV, DüMV). If threshold values for heavy metals are exceeded or if it originates from industrial wastewater, sewage sludge may also fall under the Waste Framework Directive (2008/98/EC) and has to be disposed of by incineration. The testing of sewage sludge in bioassays is not a legal requirement at present.

According to the European Waste Catalogue (2000/532/EC), waste should be characterised as hazardous or non-hazardous by considering chemical and ecotoxicological properties but no testing strategy for the criterion ecotoxicity has been defined and implemented so far (91/689/EEC, Coors et al., 2013). A list of useful ecotoxicological tests is, however, described in the guidelines CEN 14735 (CEN, 2005) and ISO 15799 (ISO, 2003) and was evaluated in an international ring test (Moser and Römbke, 2009).

On the European level, a common soil protection regulation comparable to that for the protection of water resources does still not exist. In Germany, the Federal Soil Protection Act (BBodSchG) together with the Federal Soil Protection and Contaminated Sites Ordinance (BBodSchV) stipulates that any deterioration of soil quality has to be avoided. Threshold values exist for several metals and classical organic pollutants (e.g. PAHs, PCBs, naphtaline, benzole and DDT) and the enrichment of soil with genotoxic, mutagenic, teratogenic or toxic pollutants shall be avoided (BBSchV, § 9). Alterations by agricultural usage are assumed acceptable if they comply with good agricultural practices (BBSchG, § 17).

Environmental risk assessment of chemicals

In the EU, the production and use of chemicals is controlled under the regulation (EU) No 900/2014 REACH ("Registration, Evaluation, Authorisation and Restriction of Chemicals") which addresses substances and their potential impacts on human health and the environment. The regulation applies to substances with a production or import quantity exceeding 1 ton per year, but implementation is proceeding stepwise and will not be completed before 2018. According to REACH, environmental risk assessment shall be carried out for the inland aquatic environment, the terrestrial environment and air, and the marine environment, and should proceed in the sequence of hazard identification, dose (concentration) - response (effect) assessment, exposure assessment and risk characterisation (EC, 2003).

Exposure assessment can be performed using measured environmental concentrations (MEC) or estimating predicted environmental concentrations (PEC) by the use of models (EC, 2003). For dose-response assessment, toxic properties of the substance should be identified and predicted no effect concentrations (PNECs) shall be determined. These PNECs are usually calculated by dividing the lowest short-term median effective concentration (EC_{50}) or long-term no observed effective concentration (NOEC) by an assessment factor (AF) to account for uncertainty in extrapolation from

laboratory toxicity test data to the real environment (EC, 2003). The AFs usually range from 1000 for single EC_{50} to 10 if three NOECs from species representing different living and feeding conditions are available (EC, 2003). Acute aquatic toxicity is normally determined using a fish, a crustacean and/or an algal species to cover a range of trophic levels and taxa. Terrestrial tests on plants and soil organisms are not included in the primary test set but should be conducted if a potential risk to soil via sewage sludge has been identified or can be requested for biocides and for substances with a production volume of >100 t per year or particular application to soil (EC, 2003).

Since 2003, biocide products such as disinfectants, preservatives, anti-fouling products, cooling fluids and insecticides are subject to licensing throughout Europe according to the Biocide Regulation (EU) No 528/2012. Since 2005, human and veterinary pharmaceuticals have to undergo basic environmental risk assessment for registration in the EU, too (EMEA 2006, 2008). The risk assessment differs from that within REACH by the tiered process that is followed, in which phase I considers exclusively environmental exposure while phase II, triggered by a PEC for surface waters of 10 ng/L, the environmental fate of the human pharmaceutical and its effects are considered (Oakes et al., 2010). Effects on aquatic macrophytes such as *Lemna* sp. are not required for the basic data set. Terrestrial ecotoxicity assessment is only required if the human pharmaceutical strongly sorbs to sewage sludge, as triggered by a K_{OC} >10 000 L/kg (Phase II, Tier B, EMEA 2006).

1.4. Assessing the ecotoxicological risk of complex samples

An environmental risk can be expressed in various manners, e.g. qualitatively as absence or not of a specified risk, or quantitatively as probability estimation of a risk in a specified situation (Perrodin et al., 2013). Further, the perspective can be either predictive, dealing with a potential discharge of a substance or waste, or retrospective, dealing with an existing occurrence of a substance or complex contamination (Newman, 2009). The challenge in assessing the environmental risk of complex samples is that even if a broad set of chemicals is monitored, firstly, the selection may still omit certain relevant substances, and secondly, the ecotoxicity is unknown for many micropollutants (Beyer et al., 2014, Leusch et al., 2014). Therefore, various approaches to characterise the ecotoxicity of wastewater or sewage sludge are used depending on the focus of the study and the methods available (Sarakinos et al., 2000, Newman, 2009, Perrodin et al., 2013).

Risk estimation based on single substance ecotoxicity

Research on the chemical contamination of wastewater has evolved worldwide, resulting in comprehensive data sets. Based on these data, many studies attempt to predict the ecotoxicological effects of wastewater-borne pollutants or to rank their risk for the aquatic ecosystem (Kosma et al., 2014, Valcarcel et al., 2011, Gros et al., 2010). To this aim, concentrations of single substances can

be divided by ecotoxicity data from standard biotests, i.e. median effective concentration (EC_{50}) of a specific organism, to quantify potential effects of a substance detected in an environmental sample. This quotient is mostly referred to as toxic unit (TU) (Sarakinos et al., 2000). The cumulative toxicity of the sample can then be estimated by summing up the predicted effects of the single substances at their measured concentrations to yield the sum of toxic units (sum TUs).

In principle, the sum TUs approach can be likewise applied to solid samples such as sewage sludge and the terrestrial compartment, then e.g. referred to as risk-characterisation ratios (Munoz et al., 2009, Chen et al., 2012). However, due to the lack of sediment and terrestrial effect data, it is feasible for only few substances. To overcome the lack of effect data, they can also be estimated based on aquatic effect data and partitioning assumptions based on the distribution between solid and water phase (Hernando et al., 2006, Munoz et al., 2009).

Testing the habitat quality of environmental samples

Given that chemical characterisation of treated wastewater or waste can hardly cover all pollutants present in the sample, meeting certain threshold values for priority substances does not always suffice to exclude negative impacts of the sample on the environment. Therefore, whole effluent toxicity (WET), i.e. survival, growth and reproduction of representative test organisms in effluent, has to be assessed in many countries like the USA, Australia, Japan and Europe, as summarised in detail by Power and Boumphrey (2004). Normally, a primary producer (e.g. algae), a primary consumer (e.g. crustaceans) and a secondary consumer (e.g. fish) are used to depict different trophic levels (Chapman, 2000), while in research *in vitro* tests are often applied, too (Leusch et al., 2014, Tang et al., 2014, Henneberg et al., 2014).

A number of biotest batteries has been proposed in the literature for different objectives and matrixes, mainly relating to wastewater (Persoone et al., 2003), sediments (Hollert et al., 2002, Höss et al., 2010, Kienle et al., 2013), wastes (Pandard et al., 2006), and soils, sludges and composts (Moser and Römbke, 2009). Particularly for solid matrices, the advantage of whole sample testing is that effects are integrated over all present compounds without the need to identify them and to determine their total and bioavailable concentrations (Perrodin et al., 2013). However, assessing the integrated environmental effects of treated wastewater application to soil, i.e. on both the aqueous and the terrestrial habitat, has so far only been requested in theory (Schowanek et al. 2004) but apparently not been applied to real situations yet.

1.5. Integration of the present work into the current state of research

In the following, the current state of research is summarised that was the background for the experimental studies conducted for the present thesis.

Ecotoxicity of three antimycotics and a surfactant

Antimycotics or fungicides as they are called in agriculture are applied to inhibit the growth of fungi (Zarn et al., 2002). Due to their versatile application as pharmaceuticals in human and veterinary medicines, as preservatives in cosmetics, and as biocides and pesticides, antimycotics fall under various regulations (Coors et al., 2014). One group of antimycotics among many are azoles, namely triazoles and imidazoles, containing three and two nitrogen atoms, respectively, in their heterocyclic ring of five members (Lamb et al., 1999).

Azole antimycotics are designed to inhibit the fungal lanosterol 14α-demethylase, a cytochrome P450 monooxigenase, which catalyses the formation of ergosterol (see Figure 3), the major component of fungal membranes (Lamb et al., 1999). Hence, they are also called demethylase inhibitors (Maltby et al., 2009). However, demethylation-catalysing P450 enzymes are highly conserved throughout many taxonomic kingdoms where they are involved in the biosynthesis of bulk sterols important for membranes such as cholesterol in humans and other animals and phytosterols in plants (Lamb et al., 2001, Hassold and Backhaus, 2009). Consequently, azole antimycotics have the potential to display different modes of actions in different non-target organisms.

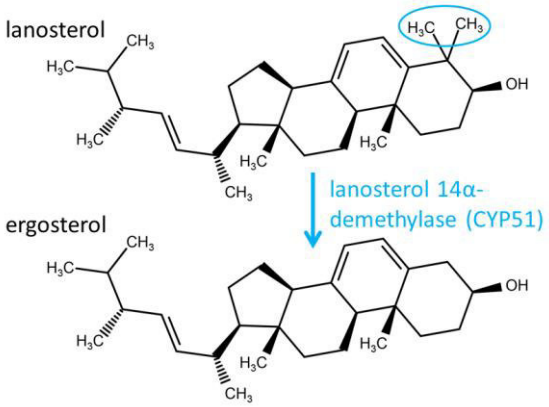


Figure 3: Demethylation of lanosterol catalysed by lanosterol 14α-demethylase.

In animals and humans, azoles may interfere with the steroid balance, i.e. the hormonal system, via the inhibition of aromatase (CYP19, precisely CYP19A1) (Lamb et al., 1999, Zarn et al., 2002, Matthiessen and Weltje, 2015). Therefore, endocrine disruption is a side effect intensively discussed in the context of antimycotic drugs in human medical treatment (Trösken et al., 2004). Likewise, not only bulk sterols but also the hormonal system can be affected in higher plants and possibly also in algae, because cytochrome P450 catalyses the first step in the conversion of ent-Kaurene into physiologically active gibberellins, i.e. phytohormones relevant for cell elongation (Kiseleva et al., 2012).

Today, azoles are detected frequently and globally in various environmental matrices (Kahle et al., 2008, Lindberg et al., 2010, Chen et al., 2013, Sabourin et al., 2011), with the actual pattern depending on their regional usage and the dominance of agricultural, municipal or hospital activities (Trösken et al., 2004). In Switzerland for example, 17 azole fungicides were registered in 2008 for agricultural use, and 14 azole antimycotics for pharmaceutical use (Kahle et al., 2008). Particularly azoles applied in antimycotics, biocides and cosmetics finally end up in municipal WWT, where they are degraded to different degrees depending on their molecular properties. While the non-degraded residues of polar azoles will stay in the water phase, i.e. in the treated wastewater, those of lipophilic azoles will rather partition to the solid phase, i.e. sewage sludge (Lindberg et al., 2010, Casado et al., 2014). For example, a Swedish research project monitoring the occurrence and fate of antimycotics during WWT found that 53% of the marketed fluconazole appears in the treated wastewater, while 35% of the marketed ketoconazole appears in the sewage sludge (Lindberg et al., 2010). Consequently, azole antimycotics enter the aquatic environment via effluent but also the terrestrial environment via the application of sewage sludge to soil, as demonstrated e.g. for climbazole, clotrimazole and miconazole (Chen et al., 2013). However, knowledge on the ecotoxicological effects of many antimycotics to assess their environmental risks is often lacking or limited to very few aquatic organisms (Hassold et al., 2009).

Against this background, three azole antimycotics, namely fluconazole, climbazole and ketoconazole, which are frequently detected in municipal wastewater (0.02 to 0.44 µg/L, Macikova et al., 2014, Wick et al., 2010, Casado et al., 2014) and sewage sludge (50 to 4450 µg/kg d.w., Garcia-Valcarcel and Tadeo, 2011, Wick et al., 2010) were selected as model substances to characterise their ecotoxicity in the present work. At the beginning of the experimental work for the present theses (in 2011), little data on their ecotoxicity was available from peer-reviewed studies.

Fluconazole, a triazole used in the systemic and topical treatment of human fungal infections against most *Candida* species (EMA, 2012), is marketed in 130 countries world-wide and in the EU is registered as product “Diflucan” and currently under revision to harmonise prescribing information (EMA, 2012). Ketoconazole, an imidazole used as broad-spectrum antifungal agent, whose oral administration for humans has been recently suspended but that remains authorised as topical antifungal agent in the EU (EMA, 2014), is characterised as potential endocrine disruptor, triggering a phase II environmental risk assessment due to be performed (EMA, 2014). Simultaneously, ketoconazole is registered as preservative in freely available cosmetics (with maximum concentration of 1% in rinse-off and leave-on products and of 2% in anti-dandruff shampoos). Climbazole, an imidazole likewise registered as preservative in topical cosmetics and personal care products such as anti-dandruff shampoos (with maximum concentration of 0.5% in rinse-off and leave-on products and of 2% in anti-dandruff shampoos), is registered under REACH with a production volume of 10 t

– 100 t/a (ECHA website, accessed 01.02.2015) and characterised as potentially persistent. In the present work, the papers in annex A.1.1 and A.1.2 focus on this topic.

Another group of micropollutants that is regularly found at high concentrations in treated wastewater and particularly in sewage sludge is the group of QACs. They are used in numerous consumer products and in the food and health care industries for cleaning, sanitizing, and disinfecting surfaces. As cationic surfactants (synonym with detergents) they reduce surface tension and form micelles, allowing dispersion in a liquid. Thus, they exert no specific antibacterial activity (as other disinfectants) but rather disturb the cytoplasmic membrane of bacteria and the plasma membrane of yeast and can also interact with intracellular targets and bind to DNA (Gerba, 2015). They inhibit growth of algae, bacteria, spores and fungi at concentrations between 0.5 and 5 mg/L, and cause lethal effects at concentrations of 10 to 50 mg/L (Gerba, 2015). Thus, they can also be applied as fungicides (Maltby et al., 2009). The mode of action of QACs appears to be adsorption to and penetration of the cell wall, reaction with the cytoplasmic membrane, followed by membrane disorganization and finally leakage of intracellular material and cell wall lysis (Gerba et al., 2015).

Due to their salt character, they form cations consisting of a central nitrogen atom with four attached organic groups, and anions usually being chlorine or bromine (Gerba, 2015). As expected by their great consumption volumes, QACs are commonly contained in wastewater (up to 0.5 µg/L) and sewage sludge (up to 25 mg/kg d.w.) of municipal, industrial and hospital wastewater (Martinez-Carballo et al., 2007, Chen et al., 2014). Among the QACs, benzyldimethyldodecyl-ammonium chloride (BDDA), the C_{12}-homologue (i.e. their main alkyl chain consists of 12 carbon atoms), is often the most prominent QAC in sewage sludge (Clara et al., 2007). BDDA is registered as existing biocide in the EU (http://ec.europa.eu/environment/chemicals/biocides/pdf/list_participants_applicants_subs.pdf, accessed 26 January 2015). Despite indications that antimicrobial activity of QACs is highest for the homologues C_{12} to C_{16} (Gerba, 2015), little specific ecotoxicity data on BDDA were available at the beginning of the present thesis. In the present work the paper in annex A.1.2 dealt with this topic.

The environmental risk of individual pollutants to terrestrial organisms is normally assessed via standard biotests, i.e. in standard soil without the application of sewage sludge. However, this neglects that the exposure will be via a sewage sludge matrix and that there may be substance-specific interaction with sludge and soil which can directly affect degradability, bioavailability and thus ecotoxicity of the substance (Eriksson et al., 2008). Hence, the transferability of standard effect data to predicting the impact at the field scale has been questioned (Schowanek et al., 2004, Natal-da-Luz et al., 2012, Golstejn et al., 2014) and is another focus of the present thesis treated in annex A.1.2.

Ecotoxicity of sewage sludge

As sorptive and poorly degradable pollutants can concentrate in sewage sludge during WWT, sewage sludge can transfer them to the terrestrial habitat when applied as soil amendment. In soil, wastewater-born pollutants can be sequestered to sludge and soil particles, can be transformed or fully degraded, can leach to groundwater, or can be taken up by plants, soil flora, microorganisms and soil fauna (Eriksson et al., 2008, Langdon et al., 2010). While in former years, concern was related mostly to the accumulation of heavy metals in soils and their uptake by crop plants (Carbonell et al., 2009), uncertainty today relates to emerging pollutants and their risks to food safety, water resources and soil quality, resulting in the ban of agricultural usage of sewage sludge in some member states or regions of the EU (Fytili and Zabaniotou, 2008, Tyagi and Lo, 2013).

Against this background, technologies in sewage sludge treatment are developed and optimised aiming at a better degradation of wastewater-born pollutants and thus a better ecological quality of the sludge (Ramirez et al., 2008). Quality in terms of ecotoxicity of sewage sludge cannot satisfactorily be assessed via the sum TUs approach because analytical methods in sludge and soil matrices are limited (Mantis et al., 2005) and terrestrial effect data are widely lacking, as discussed in detail by Schowanek et al. (2004). Therefore, assessing the ecotoxicity of sewage sludge is best approached by whole sample testing using a biotest battery (Mantis et al., 2005). A range of studies have successfully used different biotest batteries for the characterisation of single pollutants, sewage sludge quality or classification of waste (Hund-Rinke and Simon, 2004, Pandard et al., 2006, Moser and Römbke, 2009, Natal da Luz et al., 2009, Roig et al., 2012). This focus is treated in the paper in annex A.1.3.

Environmental impact of treated wastewater irrigation

Research on wastewater reuse for irrigation or aquifer recharge has focussed mainly on risks toward humans, i.e. on groundwater pollution threatening drinking water production (Cordy et al., 2004, Valcarcel et al., 2011, Postigo and Barcelo, 2015), transfer of pathogens and antimicrobial resistance genes (Dalkmann et al., 2012, Fatta-Kassinos et al., 2011, Graber and Gerstl, 2011) and on the uptake of pollutants into crops (Chefetz et al., 2008, Dalkmann et al., 2014, Grossberger et al., 2014, Prosser and Sibley, 2015).

Thus, mostly the removal of wastewater-borne pollutants during soil passage was studied (Ternes et al., 2007, Matamoros et al., 2008), whereas few attempts have been made to accommodate the risk of wastewater irrigation for the soil compartment (Munoz et al., 2009). Long-term monitoring may accompany irrigation practices and find already manifested effects retrospectively. Some cases such as former sewage farms around Berlin or Mexico City, where intensive irrigation with wastewater was practiced for almost 100 years, are well investigated regarding soil contamination (Filip et al.,

1999, Lottermoser, 2012, Dalkmann et al., 2012). However, causal links of the soil quality to the irrigation water quality cannot be drawn because land use and the composition of wastewater changed substantially over the decades. As a result, the potential environmental impact of irrigation with treated wastewater cannot be extrapolated from the existing data.

A prospective approach to assess the environmental impact can be the use of risk quotients to estimate the effects on the terrestrial habitat from concentrations of single contaminants in the effluent, as proposed by Munoz et al. (2009). However, given the scarce availability of effect data for micropollutants particularly for terrestrial organisms, this is a rather inaccurate method. Moreover, positive effects from nutrient input from treated wastewater that potentially interacts with or masks negative effects from pollutants are not considered. Thus, there seems to be a methodological gap in experimentally assessing the impact of treated wastewater irrigation on both the soil and water habitat affected. This topic was dealt with in the paper in annex A.1.4.

1.6. Objectives and hypotheses of the present thesis

The present thesis aimed at analysing the environmental risks of reusing treated wastewater and sewage sludge in land management on scales of increasing ecological relevance and with different ecotoxicological methodologies: Characterisation of the ecotoxicity of single wastewater-born pollutants; estimation of the cumulative toxicity of environmental samples based on pollutants detected and effect data; comparative assessment of the ecotoxicity of sewage sludge from advanced treatment technologies; and investigation of the integrated impact of long-term treated wastewater irrigation on water and soil habitat quality. The different research foci are graphically illustrated in Figure 4.

Depending on the objective, the bioassays were selected to represent major trophic levels and taxonomic groups of the ecosystem (climbazole ecotoxicity and effects of wastewater irrigation), to represent particularly sensitive organisms, here plants (fluconazole, ketoconazole, BDDA), or to detect differences in ecotoxicity on a screening level (sewage sludge samples).

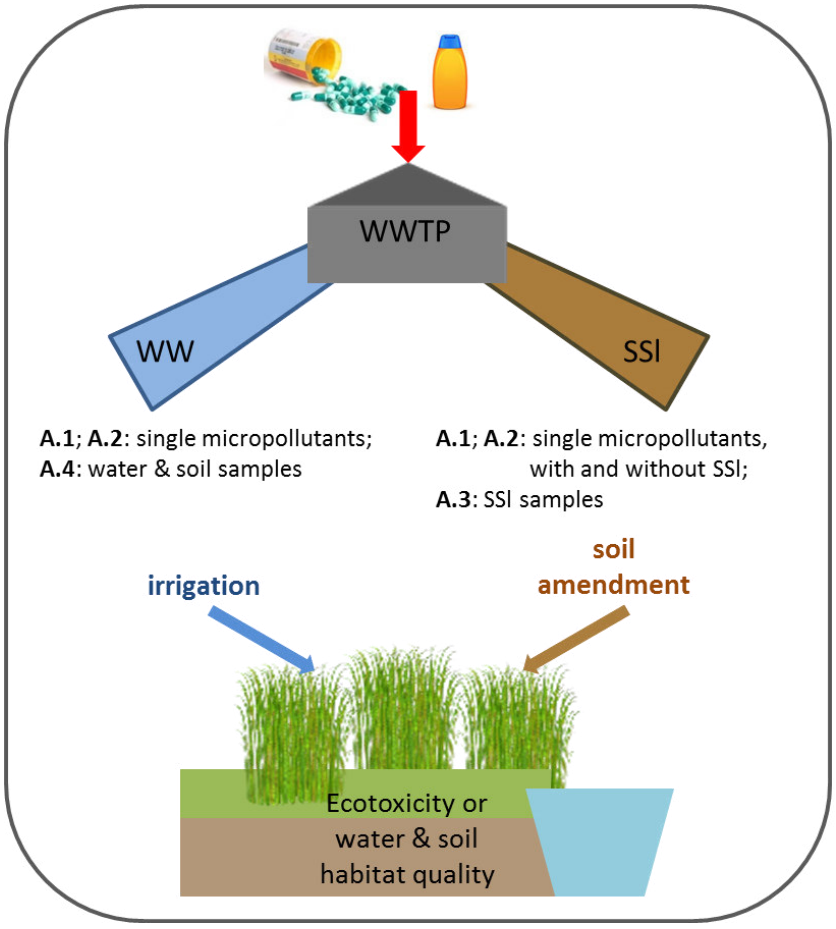


Figure 4: Research foci of the present thesis together with annex in which there are treated (A.1 to A.4). WW = treated wastewater; SSI = sewage sludge.

In detail, the objectives and hypotheses of the thesis were:

I. Characterising the ecotoxicity of the antimycotic agent climbazole:

- This antimycotic agent has similar effects on non-target organisms as agricultural azole antimycotics.
- The toxicity of the ionisable substance changes in dependence of the test soil's pH value.

To this end, the effects of climbazole toward a broad range of test organisms representative for the aquatic (green algae, cyanobacteria, water lentils, water fleas and zebra fish) and the terrestrial (oats, oilseed rape, bacteria, spring tails and enchytraeids) ecosystem were characterised. The results are described in the paper "Ecotoxicity of climbazole, a fungicide contained in anti-dandruff shampoo" (annex A.1.1).

II. Investigating the toxicity of the antimycotic agents fluconazole, climbazole and ketoconazole and the surfactant BDDA toward terrestrial and aquatic plants:

- Fluconazole, ketoconazole and likewise BDDA exert similar plant growth retarding symptoms as observed for climbazole.
- Comparing among the substances, they exert increasing aquatic and decreasing terrestrial toxicity with increasing lipophilicity of the molecule.
- The incorporation of biosolids (i.e. digested sewage sludge) alleviates effects toward oilseed rape, particularly of the sorptive substances ketoconazole and BDDA.

Based on previous findings (A.1.1) plants were expected to be particularly sensitive toward azoles and suspected to be sensitive to QACs. Therefore, the toxicity of the four test substances toward water lentils as aquatic and oilseed rape as terrestrial plant was investigated. Furthermore, the influence of simultaneous sewage sludge amendment to soil on the phytotoxicity of these pollutants was examined and results are described in the manuscript "Phytotoxicity of wastewater-born micropollutants – characterisation of three antimycotics and a cationic surfactant" (annex A.1.2).

III. Assessing comparatively the ecotoxicological quality of differently treated sewage sludges:

- Biotests allow differentiating the toxicity-reducing effects of advanced treatment methods and identification of the most suitable method.
- Observed toxic effects correlates with measured pollutant concentrations, allowing the identification of pollutants responsible for observed effects.

Novel technologies for sewage sludge digestion were developed by project partners to achieve a better quality of the final sludge. Samples of these processes were assessed by chemical analysis and two screening bioassays with terrestrial organisms (bacteria and earthworms). Results are described in the paper "Quality assessment of digested sludges produced by advanced stabilization processes" (annex A.1.3).

IV. Assessing the habitat quality of soil and water after long-term irrigation with treated wastewater of different soils:

- The model system allows predictive assessment of effects from prolonged irrigation on water and soil quality.
- Soil passage removes pollutants from treated wastewater, depending on the pollutant and on the soil characteristics.
- Passage through soils with pre-contamination deteriorates wastewater quality, particularly at the onset of irrigation.

- Soils retain sorptive wastewater-borne pollutants, particularly in carbon rich soils.
- Habitat quality of the treated wastewater is improved by soil passage while that of soil is deteriorated after the percolation as assessed by aquatic and terrestrial bioassays.
- Negative effects in bioassays correlate with those predicted by the toxic unit approach.

Effects of long-term treated wastewater irrigation on the habitat quality of soil and water were simulated applying a soil column-experiment and using chemical analyses and a range of aquatic (green algae, water lentils and water fleas) and terrestrial (oats, oilseed rape, bacteria, spring tails, enchytraeids and earthworms) bioassays. Results are described in the paper "Assessing the ecological long-term impact of wastewater irrigation on soil and water based on bioassays and chemical analyses" (annex A.1.4).

2. General discussion

In this chapter the central results presented in Annex A.1 are discussed in the context of the current state of research. To start with, the main findings of each publication are summarised. Subsequently, the methodologies applied are scrutinised for their strengths and weaknesses and the relevance of the results obtained is critically discussed. Moreover, common and alternative routes of disposing and reusing treated wastewater and sewage sludge are compared with respect to their environmental effects. The chapter ends with general conclusions and an outlook for future research.

2.1. Main findings

In the following, the main results of the present work are summarised briefly; detailed descriptions are provided in the publications in Annex A.1.

The standard ecotoxicity tests on climbazole (Annex A.1.1) revealed the following:

- The antimycotic agent climbazole is similarly ecotoxic as agricultural azole fungicides.
- Aquatic toxicity of climbazole is moderate to high for the five organisms tested, affecting them at measured concentrations of 11 mg/L and below. Median effective concentrations ($EC_{50,}$ collated in Figure 7, left) range from 11 mg/L for *Daphnia magna* (immobility after 48 hours), to 5.8 mg/L for fish embryos (mortality after 48 hours), 0.12 mg/L for the diatom *N. pelliculosa* and 0.16 mg/L for the green alga *P. subcapitata* (biomass yield inhibition after 72 hours). Among the tested organisms, the aquatic plant *L. minor* is most sensitive with an EC_{50} of 0.01 mg/L (biomass yield inhibition after 7 days).
- Terrestrial toxicity of climbazole is low to moderate for the five organisms tested (EC_{50} collated in Figure 7, right). No toxicity toward the collembolan *F. candida* and the enchytraeid *E. bigeminus* was detected in chronic reproduction tests at concentrations of up to 1000 mg/kg d.w. while for the soil bacterium *A. globiformis,* low toxicity occurs (EC_{50} of 456 mg/kg d.w. regarding inhibition of dehydrogenase activity). Plants are the most sensitive terrestrial organisms with an EC_{50} of 19 mg/kg d.w. for *A. sativa* and of 31 mg/kg d.w. for *B. napus* (inhibition of yield shoot biomass after 14 days).
- Displayed effects in plants were reduced stem elongation in *B. napus* and reduced leaf (frond) size in *L. minor*, symptoms typical for some azole fungicides and plant growth retardants. This suggests that climbazole not only interferes with the biosynthesis of bulk sterols but also specifically with the metabolism of phytohormones, presumambly gibberellins and brassinosteroids.

- Climbazole exerted increasing phytotoxicity with increasing soil pH while the influence of soil organic carbon content was comparatively low. The greater toxicity is likely related to the greater neutral fraction of climbazole at basic pH (above its pK_a).

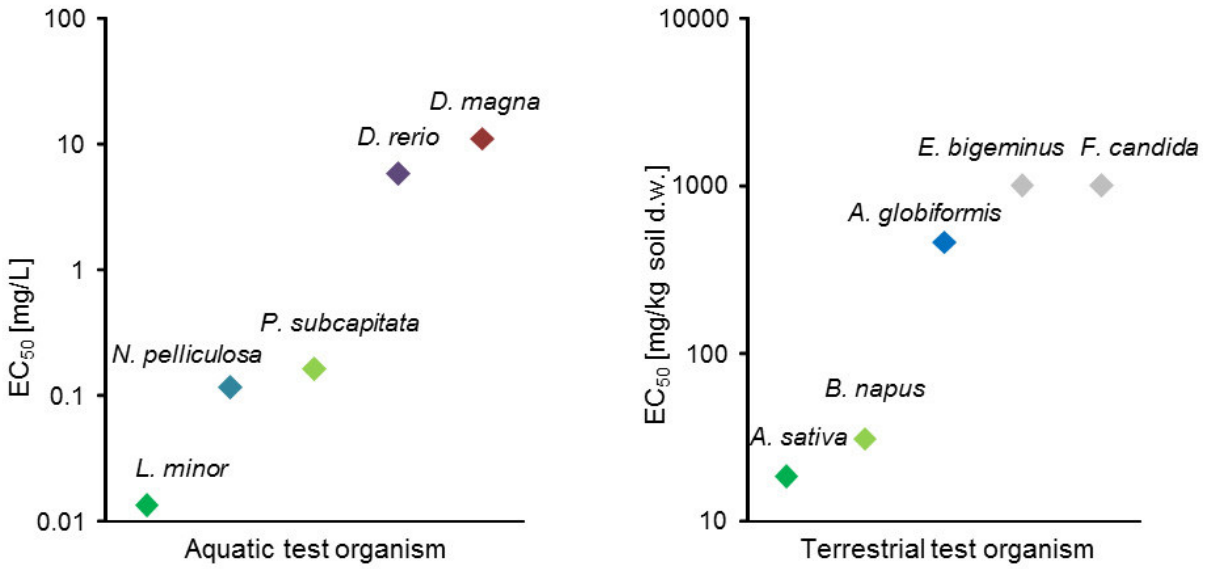


Figure 5: Median effective concentrations (EC_{50}) of climbazole in the tested aquatic (left) and terrestrial organisms (right). Aquatic EC_{50} referring to recovery corrected concentrations. In *E. bigeminus* and *F. candida*, no effects were observed at ≤1000 mg/kg d.w.

The phytotoxicity of the sludge-associated azole antimycotics fluconazole, climbazole, ketoconazole and the quaternary ammonium compound benzyldimethyldodecylammonium chloride (BDDA) (Annex A.1.2) was characterised as follows:

- All four chemicals are highly toxic toward the aquatic plant *L. minor,* whereas fluconazole and climbazole, the most hydrophilic compounds, are moderately, and ketoconazole and BDDA only slightly toxic toward the terrestrial plant *B. napus.*
- All four substances are very toxic toward *L. minor* with EC_{50} for yield frond number (recovery corrected concentrations, collated in Figure 8, left) increasing from fluconazole to BDDA, ketoconazole and climbazole with 0.97 mg/L, 0.30 mg/L, 0.06 mg/L and 0.01 mg/L, respectively.
- Terrestrial phytotoxicity increases from ketoconazole to BDDA, climbazole and fluconazole with EC_{50} (collated in Figure 8, right) of 578 mg/kg d.w., 222 mg/kg d.w., 11 mg/kg d.w. and 9.2 mg/kg d.w., respectively, regarding the most sensitive endpoint shoot biomass (fresh weight).
- One reason for the comparatively low terrestrial toxicity of ketoconazole and BDDA may be their biodegradation during the plant test which was not reflected in their EC_{50} referring to

nominal soil concentrations. Moreover, lipophilicity of ketoconazole and positive charge of BDDA probably lead to their strong sorption to soil and thus low bioavailability.

- The soil amendment with sludge (biosolids) at a realistic rate of 3.8 g/kg d.w. (corresponding to 5 t/ha) had no detectable effect on the phytotoxicity of test substances determined in soil without sludge amendment. Yet, the bioconcentration factor from soil to shoot ($BCF_{B.\ napus\ shoot}$) was found to be significantly reduced by the sludge amendment.
- Fluconazole and ketoconazole can be assumed to interfere with the phytohormone balance as they exhibited growth retarding symptoms similar to those of climbazole.
- It can be concluded that standard biotests conducted without extra sludge amendment are sufficiently realistic to assess the effects of sludge-born organic micropollutants on terrestrial plants.

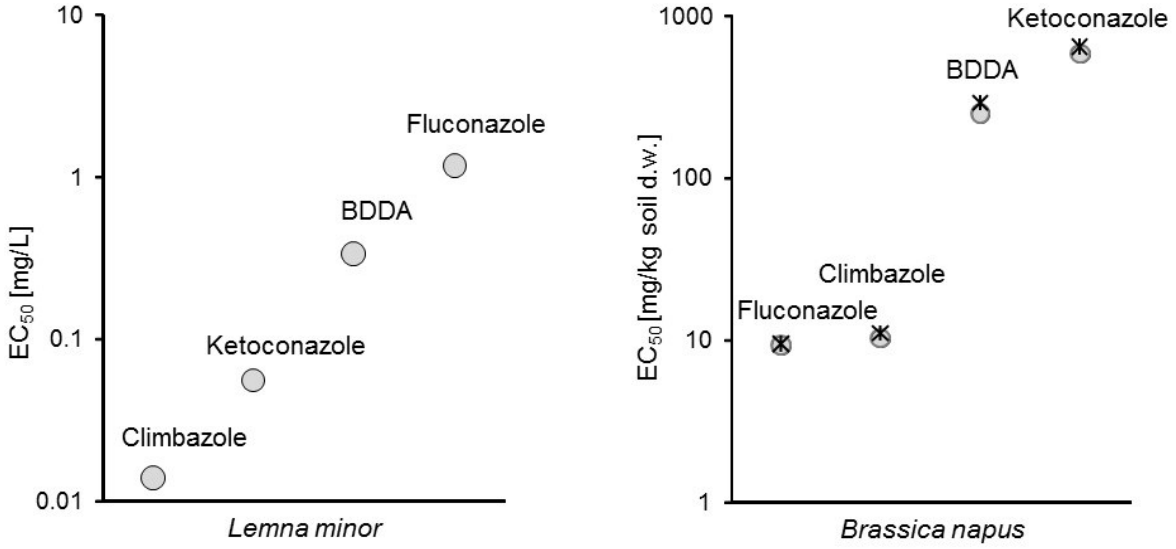


Figure 6: Median effective concentrations (EC_{50}) of BDDA, ketoconazole, climbazole and fluconazole in the plants *L. minor* (left) and *B. napus* (right). Recovery corrected values for *L. minor* regarding yield frond number; values for *B. napus* regarding shoot biomass from tests with (asterisk) and without sludge (biosolids) amendment to soil at 3.8 g/kg d.w. (grey circles).

Assessment of the ecotoxicity of differently treated sewage sludge (Annex A.1.3) exhibited the following results:

- The selected bioassays, avoidance behaviour of earthworms (*E. fetida*) and dehydrogenase activity of bacteria (*A. globiformis*), indicated differences in the quality of the sludge samples.
- All digested sludge samples showed negative effects only at concentrations (i.e. dilutions with soil) exceeding those from common European application rates (i.e. 2 t/ha with 20 cm

soil mixing depth) by at least factor 30 (*E. fetida*) and factor 100 (*A. globiformis*), respectively.

- From the detected pollutants, only concentrations of naphthalene, triclocarban and carbamazepine significantly correlated with the toxicity to *A. globiformis.* A better (negative) correlation was found between the toxicity to *A. globiformis* and the stability of the sludges (as ratio of volatile to total solids).

Evaluation of the impact of long-term wastewater irrigation on the soil and water habitat quality by means of lab-scale soil columns (Annex A.1.4) gave the following insights:

- The treated wastewater used as feed to percolate the soil columns exhibits moderate to high phytotoxicity toward *L. minor* and *P. subcapitata*. The passage through the three field soils and the reference standard soil reduces phytotoxicity, particularly in case of the two carbon-rich field soils. Neither wastewater nor percolates affect survival of *D. magna*.
- Chemical analysis demonstrates that soil passage of wastewater considerably reduces many of the wastewater-borne organic pollutants. Parallel to the reduction of phytotoxicity, the highest removal rate occurs in percolates of the two carbon-rich, peaty soils. Other wastewater-born pollutants, however, are not or only to a minor rate removed by soil passage.
- After passage through certain soils, toxicity to *P. subcapitata* increases compared to the wastewater feed. This effect is likely related to mobilisation and leaching of existing soil contamination, e.g. the metals nickel, copper and aluminium.
- The habitat quality of two field soils and the reference soil is deteriorated after the percolation with treated wastewater for at least one terrestrial test organism (deterioration compared to respective untreated soil of 73% for *E. crypticus* in soil s4, of 50% for *A. globiformis* in soil s2 and of 13% for *A. sativa* in soil s3). In contrast, the quality of two field soils improves after percolation with treated wastewater for two test organisms (improving of 26% for oats in soil s2, of 74% for *B. napus* in soil s2 and of >100% for *E. crypticus* in soil s1).
- Significantly increased soil concentrations after percolation with treated wastewater are found for zinc (up to 300%) as well as for a range of organic pollutants (e.g. benzotriazole, carbamazepine, tramadol, diclofenac, venlafaxine and metoprolol detected at >30 µg/kg d.w. in soil s3, with <5 µg/kg d.w. in untreated soils). The higher retention in carbon-rich soils compared to the sandy soils matches the higher removal efficiency observed for these soils.
- These observations underline that the impact of wastewater percolation on the soil quality depends on the interaction of soil characteristics, pollutant properties and test species-specific

sensitivity. In general, high removal efficiency of a soil, i.e. low leaching potential of wastewater-borne pollutants to groundwater, can come with a high retention of pollutant residues in soil and thus the deterioration of soil habitat quality.

2.2. Usefulness of the applied methodologies

In the following chapter, the methodologies applied in the present work for testing and evaluating ecotoxicity are scrutinised.

Single substance testing approach

Characterising the ecotoxicity of a substance as requested for its marketing authorisation follows well established and recognised test guidelines with standard test organisms. The aim of these bioassays is to investigate the quality of effects and to establish a concentration-response curve between an effect and a series of concentrations. This was the main objective of the bioassays with climbazole (A.1.1) and with fluconazole, ketoconazole and BDDA (A.1.2).

Technical difficulties can arise when testing sorptive substances. Measured concentrations in aqueous test solutions should normally range within 80% - 120% of nominal concentrations (OECD 23, 2000) in order to maintain stable concentrations during the test. However, this was not the case for BDDA and ketoconazole in the *L. minor* tests (A.1.2). For BDDA, a preliminary test had demonstrated high losses during the test so that a semi-static test design with two intermittent renewals of test solutions was chosen for the final test (A. 1.2). Nevertheless, the overall recovery was only 44% of the nominal concentrations. For ketoconazole, its low water solubility necessitated the use a solvent for the preparation of the stock solution. A semi-static test design would have been advisable but could not be accomplished in the present work due to project-related time scales. To what degree the low recovery of BDDA and ketoconazole was due to sorption or to degradation could not be elucidated by the limited sampling scheme. However, it can be assumed that degradation at least contributed as supported by literature on their degradability in WWTPs (Zhang et al., 2011, Peng et al., 2012).

In terrestrial tests, recovery analysis of test concentrations is usually not requested because the soil matrix itself renders chemical analyses laborious and recoveries in soil are usually rather low (Barron et al., 2008, Peyson and Vulliet, 2013). Moreover, field exposure will usually originate from point sources such as pesticide or sludge application, so that the administration of test substance only at test start and a potential decline in soil concentrations during the experiment can be regarded as realistic scenario. Yet, especially in case of sorptive or degradable substances, the nominal EC_{50} certainly underestimates their terrestrial toxicity as will be true for ketoconazole and BDDA (A.1.2).

Another critical issue in standard plant testing (OECD 208, 2006a) applied in the present work (A.1.1 and A.1.2) is that it does not simulate contaminant exposure from irrigation with treated wastewater. From irrigation, the exposure will rather be continuous with low but constant concentrations that would be better covered by e.g. assessing plant growth in semi-static hydroponic cultures (Tanoue et al., 2012). This test design, however, was out of the scope of the present work.

Another important aspect in the ecotoxicity assessment of azoles, which are usually weak bases, is their pH dependent ionisation (see A.1.1 and A.1.2). The relationship between the charge of the molecule and its toxicity can be explained by various models (Escher et al., 2006, Kah and Brown, 2007). Yet, estimating the neutral molecule fraction present at the very site of uptake into the organism is not trivial because the pH usually shifts during the bioassay and depends on the location of measurement in the test medium (Rosenkrantz et al., 2013, Altenburger et al, 2010). For example, the pH probably differs between the test medium and the cytosol of algae and water lentils, or the bulk soil, the rhizosphere, the cytoplasm and vacuole of plants (Neuwoehner and Escher, 2011, Trapp, 2000). Moreover, finding a buffer to maintain a stable pH during the test that does not interact with the test substance or inhibit the performance of the test organism is necessary (Altenburger et al., 2010, Rendal et al., 2011, 2012). However, refined measurement of pH gradients in the test medium and soil were beyond the scope of the present work.

In terrestrial ecotoxicity testing, the standard procedure of applying test substance to standard soil for simulating contaminant exposure via sewage sludge can be questioned. It was suggested that toxicity can be severely influenced by the organic carbon content of the soil (Golstejn et al., 2014) and by the addition of extra carbon from sewage sludge or biosolids (Schowanek et al., 2004). Therefore, the biosolids amendment of test soil in the present work aimed at assessing the differences between standard tests and this realistic exposure scenario (A.1.2). Yet, the complexity of a realistic exposure could not be covered in the present test design. For example, the method of spiking the test substances to the sludge is crucial for the translation of exposure concentrations into effects as demonstrated e.g. for the extractability of metals in soil (Natal da Luz et al., 2011. Moreover, aging of the spiked biosolids plays a role as it can lead to enhanced sequestration of pollutants in the solids (Rogers 1996). Further, the biosolids to pollutant ratio also determines the influence on effects; it will probably be higher at a high sludge to pollutant ratio as also found for the transport behaviour of pharmaceuticals in biosolids-amended soil (Borgman and Chefetz, 2013).

Cumulative toxicity estimation approach

As explained in chapter 1.4, the ecotoxicity data of single-substance bioassays as retrieved in A.1.1 and A.1.2 can be used to estimate the cumulative toxicity of environmental samples. For industrial effluents, where contamination is characterised by few substances at high concentrations, predicted

effects based on the sum TU approach can be in good accordance with observed effects (Sarakinos et al., 2000). Likewise, the sum TUs method has been used for assessing the environmental risk of municipal wastewater, with a focus on pharmaceuticals (Hernando et al., 2006, Gros et al., 2010, Valcarcel et al., 2011, Kosma et al., 2014) and pesticides (Köck-Schulmeyer et al., 2013). However, the risk estimations obtained in these studies have not been confirmed by experimental evidence. Moreover, care has to be taken in comparing risk quotients with TUs, as risk quotiens are rather conservatively estimated based on PNECs (Hernando et al., 2006), while TUs are based on EC_{50} data. Thus, a TU >1 would indicate a detrimental effect of 50% or in 50% of an exposed cohort, whereas a risk quotient >1 would indicate that a risk toward the ecosystem cannot be excluded (Hernando et al., 2006). Generally, applying the sum TUs approach to predict effects of a complex sample has limitations as described for the effluent and percolate samples in A.1.4:

Firstly, characterising the environmental pollutant exposure in terms of quality (which substances?) and quantity (what concentration?) will always be connected with uncertainty because even the most thorough analysis can account for only a fraction of all potentially present chemicals and thus may ignore toxicity relevant substances (Valcarcel et al., 2011, Magdeburg et al., 2014, Tang et al., 2014). If non-target analysis is used as supplement to screen for unknown substances and transformation products, identifying and quantifying the candidates remains sophisticated and laborious (Munoz et al., 2009, Wick et al., 2010, Krauss et al., 2010). Analysis in different environmental matrices involves identification and quantification of the respective substances in complex media with matrix properties that often influence the analytical method used, such as organic carbon content or high salinity (Rogers, 1996, Daughton 2004, Peysson and Vulliet, 2013). To eliminate matrix effects, extraction and concentration steps and the selection of solvents prior to instrumental analysis are often required (Barron et al., 2008, Wick et al., 2010, Casado et al., 2014). Thus, chemical analysis of environmental samples is always selective, depending on the choice of pollutants to be analyzed as well as their detection limits (Valcarcel et al., 2011). Also the characterisation of environmental water samples is not unambiguous because concentrations can either refer to the dissolved substances, i.e. determined in filtered samples, or include particle bound substances, i.e. determined in unfiltered samples, which is usually much greater for sorptive substances such as BDDA and other QACs (Kreuzinger et al., 2007).

Secondly, the availability of effect data for detected substances is limited particularly with regard to pharmaceuticals and ingredients of personal care products (Gros et al., 2010, Munoz et al., 2008, von der Ohe et al., 2011, Kosma et al., 2014) as discussed for the wastewater percolates in A.1.4. This general lack of ecotoxicity data for micropollutants is unlikely to be overcome in view of the continuous development of new chemicals and active substances (Valcarcel et al., 2011). Available effect data are mostly restricted to acute toxicity to algae, daphnia and fish (Gros et al., 2010).

Studies on other organisms or endpoints often possess little reliability because their methodology is insufficiently reported, not freely available, and not comparable to other studies (Munoz et al., 2008). Moreover, particularly heavy metal toxicity is hard to quantify under different environmental conditions as their speciation and complexation varies with the physico-chemical parameters of the medium (Florence, 1982, Birsan and Luca, 2010). Despite the long history of intense research on the fate and effects of heavy metals (Allen et al., 1980, Seco et al., 2003, Smolders et al., 2009, Stravinskene and Grigorev, 2012) and many conceptual approaches to model their effects in dependence of physico-chemical properties by biotic ligand models, validated modelling tools are available for only a few metals and organisms (Paquin et al., 2002, Erickson, 2013). Therefore, and because only a limited set of water parameters was available for all samples, the sum TUs calculation in A.1.4 could not adequately account for the quantitative influence of heavy metals on the observed effects. Not reflected in the sum TUs approach is further the interaction of different pollutants with each other and with other medium constituents such as nutrients.

Thirdly, the correlation of total measured concentrations with observed effects may exist for the aquatic samples (Sarakinos et al., 2000) but rarely for soil and sediment samples, because the substances may be unavailable for uptake (e.g. due to strong adsorption onto clay or organic particles or by sequestration inaccessible soil pores) (Didden and Römbke, 2001, Sarkar et al., 2010). Therefore, the sum TUs approach has rarely been applied for solid samples such as soil. Munoz et al. (2009) propose to use risk-characterisation ratios to assess the ecotoxicological risk of wastewater irrigation toward the terrestrial ecosystem; yet, most terrestrial effect data are modelled from aquatic effects so that this approach is linked to some uncertainty.

In conclusion, the results of the present work add further evidence that assessing the cumulative risk via the sum TUs approach is currently insufficient to describe or predict environmental effects as occurring in treated wastewater and sewage sludge.

Environmental sample testing approach

By subjecting an environmental sample as a whole to bioassays, the environmental risk of all pollutants present in the sample and their possible interaction is taken into account, irrespective of knowledge of their identity and quantity. Thereby, “whole sample” testing improves the realism of the risk assessment compared to single substance testing and the sum TUs approach so that the findings possess high ecological relevance. Yet, it has to be noted that not the absolute ecotoxicity of an environmental sample is determined, but its habitat quality for a certain organism (Moser and Römbke, 2009, Feiler et al., 2013) and that habitat quality integrates over physical, biological and chemical properties of the sample, so that toxicants are only one among other factors. However, as mentioned for the TUs approach, chemical analyses alone are likewise selective as they depend on

matrix characteristics (e.g. organic carbon content, salinity) and on analytical techniques applied (e.g. extraction method and solvent type) (Barron et al., 2008, Daughton 2004, Peysson and Vulliet, 2013).

The selection of bioassays for a test battery generally depends on the objective: If the environmental hazard of a sample to the receiving ecosystem is assessed, bioassays will be chosen to investigate potential effects on important taxonomic and functional organism groups of this ecosystem. Crucial, however, is to warrant that the biotest system or organism is not inhibited by the matrix properties, and if so, that this effect is accounted for by normalising results of a test soil to an appropriate control soil or medium (Hund-Rinke and Wiechering, 2001, Aruoja et al., 2004). For interpreting the results, a certain level of effects has to be agreed upon to be acceptable (Moser and Römbke, 2009). In contrast, if changes in the ecotoxicity of a sample with roughly known pollution are to be assessed, then bioassays will be selected that are known to respond to the suspected pollutants in the given concentration range (e.g. water lentils to herbicides or molluscs to endocrine disrupting pollutants) (Kienle et al., 2013, Magdeburg et al., 2014).

A further drawback is that in the absence of effects, results cannot be extrapolated to other time scales, sample dilutions or organisms. To overcome this problem, aqueous samples can be concentrated via solid-phase extraction as it is usually done for *in vitro* testing (Magdeburg et al., 2014); yet, by this procedure many polar substances may be ignored.

Overall, results from whole sample testing have to be interpreted context specific and care has to be taken when transferring conclusions to other sites and samples (Moser and Römbke, 2009).

Representativeness of selected bioassays

Bioassays used in the present work did not attempt to find the most sensitive test organism for each individual sample set but generally followed standardised test protocols to attain comparable and representative observations. Applied test organisms are illustrated in Figure 9.

The bioassay selection for measuring the ecotoxicological quality of sewage sludge and for comparison among differently treated sludge samples (A.1.3) was guided by literature recommendations on testing of sludge (Natal da Luz et al., 2009) and waste (Moser and Römbke, 2009, CEN 2011), but was simultaneously restricted by the volume and numbers of samples received. Therefore, two small-volume and short-term tests were selected with soil organisms representative for soil fauna and microflora exposed to biosolids amendment, namely earthworms and bacteria.

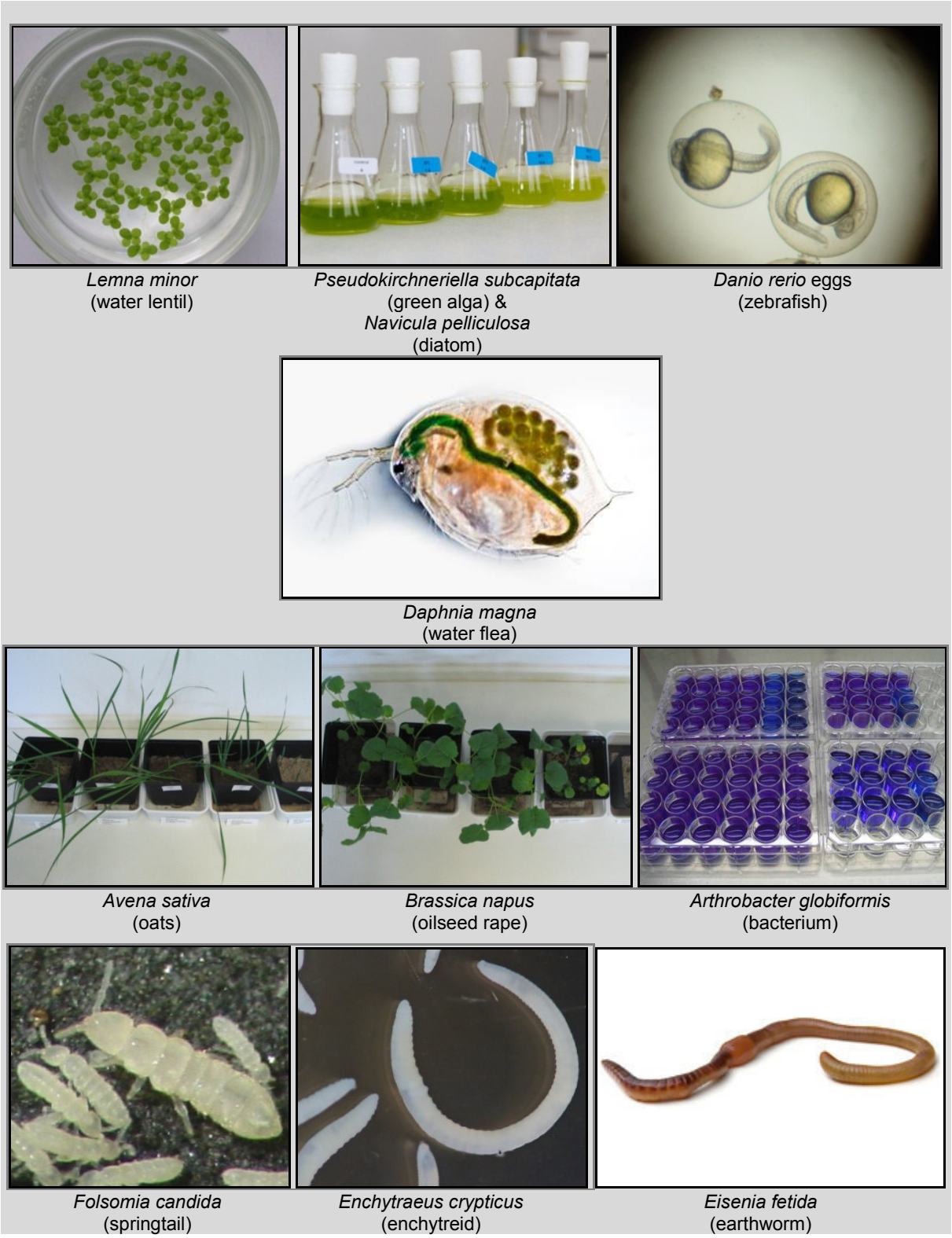


Figure 7: Illustration of test organisms used in the bioassays of the present work. Photos by ECT.

Inhibition of dehydrogenase activity in *A. globiformis* can be seen indicative for early and subtle effects in soil microflora as the bacterium is one of the most commonly found strains in soils (Rönnpagel et al., 1998). Moreover, *A. globiformis* is representative for many bacteria genera as it is

aerobic facultative anaerobic, thus being able to live in soil and sediment, and gram-positive gram-variable, thus integrating over gram-positive bacteria (which are e.g. especially sensitive toward heavy metals) and gram-negative bacteria (which perform nitrification) (Rönnpagel et al., 1998). Still, inhibition of enzyme activity is not a lethal endpoint so that effects on the soil microbial community can potentially recover from temporal pollutant exposure (Carbonell et al., 2009).

Earthworm avoidance testing is suitable for small sample volumes and high throughput of samples, and as it refers to a key organism for terrestrial litter decomposition, the results have high ecological relevance (Moser and Römbke, 2009). Avoidance by ≥80% is defined as the threshold value that indicates a limited habitat function for the test soil (ISO 17512-1, 2010). Avoidance behaviour, in contrast to mortality or reproduction, is not a standard endpoint within chemical risk assessment, but can be used for screening as it was demonstrated to be more sensitive than acute earthworm mortality (Moser and Römbke, 2009) and similarly sensitive as reproduction for many chemicals (e.g. Cd, Cu, PCP, TBT, TNT) (Hund-Rinke et al., 2005, Natal de Luz, 2004). However, not all contaminants seem to induce avoidance in earthworms (Reinecke et al. 2002). Therefore, these two tests are suitable as screening tests but for reducing uncertainty, also chronic bioassays should be performed to assess long-term impacts from sludge amendment to soil.

The terrestrial bioassays selected for testing the wastewater percolated soils (A.1.4) are well adapted to the assessment of contaminated soils and wastes (Moser and Römbke, 2009, Jänsch et al., 2005). As recommended for the assessment of soil quality (ISO 15799, 2003), the applied tests cover different trophic levels important for the functioning of the ecosystem (primary producers, destruents and decomposers) and important taxonomic groups (mono- and dicotyledonous plants, bacteria representing soil microflora, annelids and arthropods representing soil mesofauna).

Negative effects from the percolation with treated wastewater were observed on *A. globiformis*, *A. sativa* and *E. crypticus* in different soils. Enchytraeids which belong to Annelida, Oligochaeta, play a key role in the functioning of soil ecosystems, mainly processes such as decomposition and bioturbation of organic matter, are present in a wide range of ecosystems and come into contact with the solid, the gaseous as well as the aqueous phase of the soil. Therefore, enchytraeids are representative for many soil organisms and are used as indicator organisms for environmental stress (Didden and Römbke 2001). Plants as primary producers are indispensable for a functioning ecosystem, comprising soil microflora such as algae as well as higher plants. In contrast to soil fauna that are exposed to pollutants via dermal contact with soil solution and ingestion of solids, higher plants are able to actively change their close environment (rhizosphere) by the excretion of acids or chelates which shall enhance nutrient supply but may also facilitate pollutant uptake. Moreover, their fine root hairs intensively penetrate the soil so that the exposed root surface is very large (Trapp, 2000). Translocation of pollutants from roots to the shoot seems to be most efficient for compounds

of intermediate polarity (Briggs et al., 1982) because highly hydrophilic compounds are less able to cross hydrophobic membranes, while highly lipophilic compounds are retained in the root lipids (Tanoue et al., 2012).

Whole effluent toxicity testing (WET) bioassays are widely applied and often used to monitor the quality of industrial waste water (Sarakinos et al., 2000, Warren-Hicks et al., 2000, de Vlaming et al., 2000, Maltby et al., 2000). Strategies, benefits and limitations of WET have been intensely discussed over some decades (Power and Boumphrey, 2004). Chapman (2000) reviewed WET and points out that they may be overprotective (due to their conservative design, the absence of exposure alleviating processes, and sensitivity to non-contaminant effects), underprotective (due to more sensitive species and endpoints, occurrence of multiple stresses in receiving environment, and food chain effects), or offer an uncertain level of protection (intermittent doses and mixtures in the environment, adaptations, and hormesis). Therefore, Chapman (2000) concluded that WET tests should not be seen as precise predictors of effects in the receiving surface water but rather as indicators identifying hazards. The same should be true for the present findings (A.1.4). As pre-tests with treated wastewater in the present work had shown negative effects on algae but no toxicity to fish embryos, the latter test was removed from the test battery and a test on water lentils was added. Assessing acute toxicity to *D. magna* was included in the biotest battery for the wastewater percolation experiment because crustaceans could have indicated heavy metal leaching from the soils as was observed for leachates of metal containing wastes (Seco et al., 2003).

To answer the question which bioassays are suitable for assessing the ecotoxicological effects of effluent irrigation on water and soil, a number of trials have to be repeated with the same bioassays, soils and effluent source. Then, the effects compared to the response variance of each test could be better evaluated and recommendations given tailored to fit the needs of the application (Magdeburg et al., 2014, Margot et al., 2013, Escher et al., 2014). For example, redundancy of test results could be analysed via multivariate analyses (Pandard et al., 2006) in order to define a limited set of bioassays with optimal value of information. Based on the present study, no reduction but rather an inclusion of further bioassays to detect substances with specific modes of action appears necessary. *In vitro* bioassays for example can detect specific modes of action at low concentration levels (usually after sample enrichment via solid phase extraction) and with high throughput. Yet, they are not available for all modes of action, they are unable to accurately predict whole organism effects and lack environmental relevance and regulatory acceptance (Leusch et al., 2014).

Evaluation of results from different approaches

Effects from single substance testing are usually described quantitatively, i.e. as percent inhibition at a given concentration in relation to the response in the control medium (e.g. by EC_{50}), or

qualitatively, i.e. as statistically significantly different response of organisms exposed to a test concentration compared to organisms in a control medium (e.g. by NOECs). In the present work, EC_x were determined to characterise the toxicity of the investigated antimycotics and BDDA (A.1.2) and of the sludge sample quality (A.1.3), to compare toxicity among substances or samples, and to relate effects to measured environmental concentrations (MECs) for a preliminary environmental risk assessment.

Assessing the quality of environmental samples mostly aims at determining whether or not the sample differs from that of a control or reference substrate, as done for the sludge sample effects to *E. fetida* (A.1.3) and for the water and soil samples before and after wastewater percolation through different soils (A.1.4). In the context of effluent and waste testing, however, an effect can also be regarded significant by exceeding a specified magnitude of inhibition, so called toxicity thresholds or toxicity criteria (Römbke and Egeler, 2009, ISO 17616, 2008, Höss et al., 2010). Results are often expressed in Lowest Ineffective Dilution values (ISO, 2006). These values have been defined for each test system separately on the basis of experience and round robin tests (ISO 17616, 2008, Moser and Römbke, 2009, Höss et al., 2010), and are e.g. specified as 20% for the acute *Daphnia* test, 25% for *Lemna* and algae, 30% for the plant test and bacteria tests and 50% for the reproduction tests with earthworms, collembolans and enchytraeids (ISO 17616, 2008) or 80% for the earthworm avoidance test (ISO 17512-1, 2010). For the assessment of natural sediments, toxicity thresholds of 60%, 25% and 20% were proposed for enzyme activity of bacteria (*A. globiformis*), reproduction of oligochaetes (*Lumbriculus variegatus*) and growth of macrophytes (*Myriophyllum aquaticum*), respectively, based on evaluation of the minimum detectable difference per test (Höss et al., 2010).

Applying these threshold values for the effects on habitat quality observed in the soils after percolation with treated wastewater (A.1.4), the 13% deterioration for plants in soil s3 and the 26% improving for plants in soil s2 can thus be regarded as not ecologically relevant, despite the statistically significant difference compared to the respective untreated soil. In contrast, the deterioration of habitat quality found for bacteria in soil s2 (50%) and for enchytraeids in the reference soil s4 (73%) clearly lie above the respective threshold values. This underlines the ecological relevance of the detected habitat deterioration of the peaty soils by wastewater percolation.

2.3. Relevance of retrieved findings

In the following, the environmental relevance of the findings retrieved in the present work will be discussed.

Environmental relevance of tested substances

The antimycotics climbazole, ketoconazole and fluconazole and the surfactant BDDA were shown to be highly phytotoxic having chronic aquatic effective concentrations in the µ/L range. Compared to available data on concentrations measured in the environment (A.1.1 and A.1.2), no immediate risk appears to be indicated; yet, the margin of safety under the described conditions is narrow. Among the four test substances, the greatest risk for the aquatic compartment seems to be connected to climbazole, while for the terrestrial compartment the greatest risk can be expected for BDDA.

Growth of primary producers appears to be a very sensitive endpoint for the azole antimycotics, as discussed in A.1.1. In Figure 10, reduced stem elongation, curled leaves and growth retardation in *B. napus* resulting from fluconazole treatment is illustrated, i.e. symptoms which are typical for the inhibition of gibberellin and brassinosteroid phytohormones (Rademacher, 2000, Rajalekshmi et al., 2009, Oh *et al*., 2012.

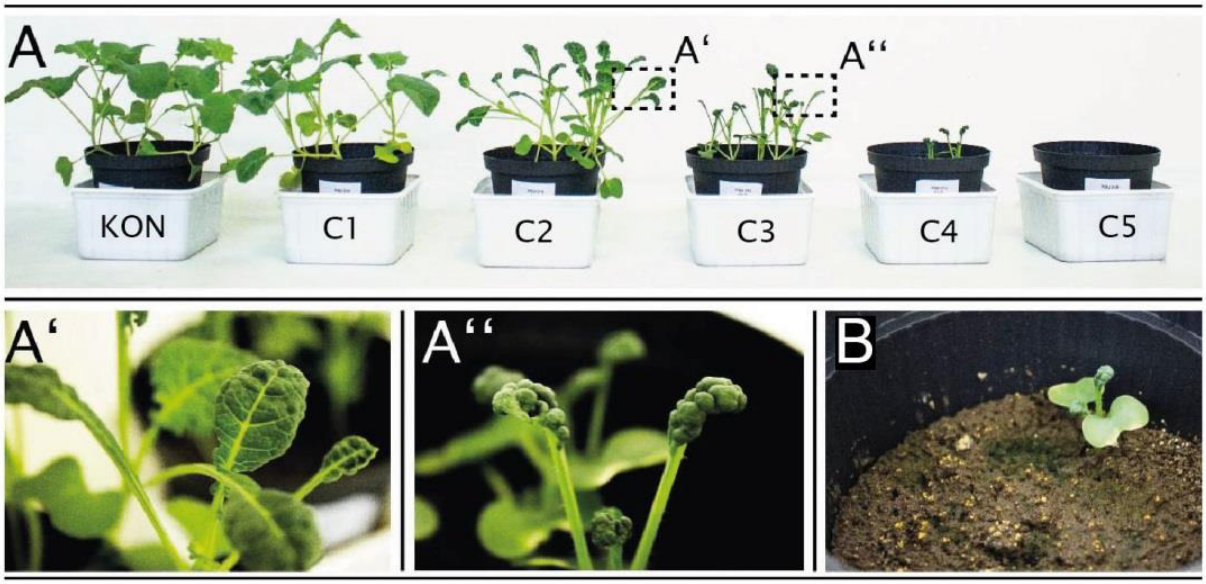


Figure 8: Morphological effects of fluconazole on *B. napus* at test end. A: Treatment concentrations ranging from 1-81 mg/kg soil d.w. (C1 to C5), KON = control. A': leaf at 3 mg/kg soil d.w.; A'': leaf at 9 mg/kg soil d.w.; B: plant at 81 mg/kg soil d.w; (from Richter et al., submitted, A.1.2).

While organisms such as *D. magna* or fish embryos seemed to be less sensitive than plants at the short term time scale (A.1.1), the reproduction of crustaceans and molluscs has been demonstrated to be affected at similar azole concentrations as plant growth (Hassold and Backhaus, 2009, Janer et al., 2006). This may be explained by the existence of two modes of action of azoles that lead to the observed effects in both plants and animals: acute effects linked to the disturbance of bulk sterols important for membrane stability, and chronic, more sensitive and more substance-specific effects linked to the disturbance of phytohormones and the endocrine system (Crane et al., 2006, Lamb et al., 2001).

Interestingly, the fungicidal effect of azoles seems to be related not to their specificity for fungal CYP enzymes but rather to the availability of these enzymes in the organism. This hypothesis is supported by the observation that the fungicides triadimenol and tebuconazole are only threefold more effective against purified CYP51 from fungi compared to that from plants (Lamb et al., 2001). While fungi possess rather few and thus more essential CYP51 enzymes, the multiplicity of CYP51 enzymes in plants can probably better mitigate negative inhibitory effects (Lamb et al., 2001). Thus, the usage-specific classification of an azole as agricultural pesticide, antifungal drug, or antiestrogenic agent conceals their common mode of action (Trösken et al., 2004) and their potential hazards to the environment as well as to humans.

Also indirect effects can be expected from azole antimycotics, e.g. leaf litter breakdown by aquatic shredder organisms (Zubrod et al., 2011). Via changing the fungal species composition on the leaves, the fungicide tebuconazole affects *Gammarus fossarum* already at µg/L concentrations (Zubrod et al., 2011). While these effects are not investigated regularly they let assume that also other ecosystem functions may be disturbed at trace azole concentrations. Hence, current environmental risk assessment that does not require testing fungi or macrophytes may provide insufficient protection for azole antimycotics and fungicides.

As azoles are ionisable substances, their toxicity is dependent on their degree of ionisation because the membrane permeability of the charged molecule is assumed to be by orders of magnitude lower than that of the neutral compound (Rendal et al., 2012). Ionisation follows a logarithmic curve; hence, a small change in the pH of the test medium can strongly affect the percentage of neutral molecule (Rendal et al., 2012). This is illustrated for the weak bases climbazole and ketoconazole (Figure 11) getting more and more cationic at acidic pH, i.e. below their acidity constant (pK_a), the inflection point of the curve at which 50% of the molecules are neutral. In contrast, BDDA is permanently cationic in aqueous solution, whereas fluconazole with pK_a values at 2.5, 2.9 and 11.0 (Correa et al., 2012) is a zwitterion that is neutral at environmentally relevant pH ranges.

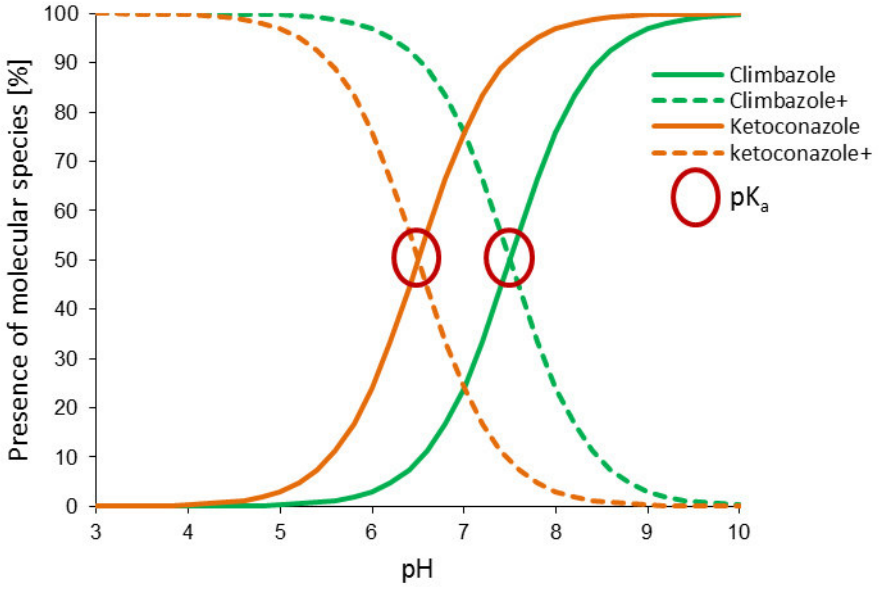


Figure 9: Partitioning of climbazole and ketoconazole into neutral and cationic molecules depending on the pH of the test medium.

In case of climbazole, 50% of the molecules are neutral at pH 7.5 (climbazole' s pK_a) while at pH 6.5 and 5.5., a marginal percentage (9% and 1%, respectively) are uncharged according to the Henderson-Hasselbach equation (see A.1.1). As demonstrated in A.1.1, phytotoxicity of climbazole is indeed significantly higher at basic soil pH, presumably due to the lower fraction of the strongly sorbing cation. Results retrieved from standard bioassays at a specific pH may thus not be representative for nature, where the pH in surface waters can fluctuate between pH 6 and 9 (Rendal et al., 2011, Oaks et al., 2010). The pH of natural soils may cover pH 3 to 10, with agricultural soils in humid regions ranging mostly between pH 4 to 7 (Scheffer/Schachtschabel, 2010).

Regarding the fourth model substance, the QAC BDDA, little data on ecotoxicity can be found, especially for terrestrial organisms (Kreuzinger et al., 2007). However, Kreuzinger et al. (2007) came to the conclusion that at least for aquatic and sediment dwelling organisms, risks from QACs cannot be excluded.

In the preliminary environmental risk assessment conducted in the present work, the test substances were assessed on an individual level (A.1.1, A.1.2). Yet, together with the tested azoles and BDDA there occur many other azoles and QACs sharing similar structures and modes of action in surface water or sewage sludge (Kahle et al., 2008, Trösken et al., 2004, Kreuzinger et al., 2007). Besides households, agricultural practice can be a source of fungicides (Ochoa-Acuna et al., 2009) whereas QACs can enter surface water additionally from the effluent of paper mills or swimming pools (Gerba, 2015). Toxicity of a mixture of pollutants can mostly be well predicted by the concentration-addition concept (Coors et al., 2014, Spurgeon, 2010). However, azoles may display different

mixture toxicity patterns in combination with pyrethroid insecticides. On the one hand, high concentrations of azoles potentiate the toxicity of pyrethroids by inhibiting their detoxication via monooxigenases (enzymes responsible for the phase I metabolisation of lipophilic compounds) (Cedergreen, 2014). On the other hand, at low concentrations azoles seem to induce the activity of monooxygenase P450, thus alleviating the toxicity of pyrethroids (Cedergreen, 2014). Similarly, growth stimulating, hormetic effects were observed for low concentrations of a mixture of pharmaceuticals and personal care products (fluoxetine, propranolol, triclosan, zinc-pyrithione, and clotrimazole) to marine periphyton (Backhaus et al., 2011). Thus, predicting effects of azole antimycotics present in treated wastewater or sewage sludge as sum of their individual toxicity is related with uncertainty as it may over or underestimate their risk to the environment.

Also surfactants such as the QAC BDDA may interact with other pollutant groups. On the one hand, they can promote the uptake of other organic pollutants through biological membranes thereby creating a toxicokinetic synergistic interaction (Coors et al., 2014). On the other hand, the toxicity of the cationic QACs can be reduced in nature by the presence of anionic detergents due to neutralising their binding strength (Kümmerer et al., 2004). Further, QACs have also been found to stimulate microbial activity in soils when present at low concentrations (Sarkar et al., 2010). Hence, the effects of BDDA detected in an environmental sample of complex composition may differ from its toxicity estimated simply on its measured concentration.

Another concern related to environmental pollution is the evolution of resistance of the target organisms to the respective drugs or biocides. However, despite the widespread use of antimycotics and fungicides, the potential of fungi for developing resistance appears to be comparatively low. Only for fluconazole, the selection of fluconazole resistant *Candida* spp. strains among clinical isolates seems to be of increasing importance (Hof, 2008). Similarly, the development of resistance against QACs like BDDA seems rather unlikely considering their mostly nonspecific action and multi-target nature (Gerba, 2015). However, QACs can induce membrane efflux pumps that in some cases help bacteria to gain adaption against certain antibiotics. Thus, facing the widespread use of QACs, the development of bacteria with decreased sensitivity to antibiotics is of current concern (Gerba, 2015).

Relevance of findings for land application of sewage sludge

As found in the present work and discussed in A.1.4, it seems valid to assess effects on terrestrial plants of sludge-associated pollutants by standard bioassays, i.e. without adding sludge to the test substrate. Moreover, it was observed that sludge treated by advanced technologies had a comparable ecotoxicological quality as conventionally treated sludge and that its toxicity rather depended on its stability than the concentrations of single pollutants (A.1.3). Similar findings are reported for effects

of differently treated sewage sludge on plant root elongation and bacteria luminescence (Roig et al., 2012) or plant growth (Ramirez et al., 2008) which did not correlate to total heavy metal or organic pollutant concentrations of the solids. This can probably be attributed to the strong binding of pollutants to the solids so that total measured concentrations give little information on bioavailable, toxicity relevant concentrations. From literature and from the findings of the present work (A.1.3) it can be concluded that stability of municipal sludge is of greater influence on sludge ecotoxicity than the total pollutant load. Consequently, sludge stabilisation processes such as advanced anaerobic-aerobic combined treatments or further composting appear to be more effective for the reduction of ecotoxicity than optimising degradation of single pollutants on a marginal scale.

All the digested sludges investigated in A.1.3 posed no immediate ecotoxicological risk to the test organisms, with a safety margin between concentrations that caused effects (50% and >80% for *A. globiformis* and *E. fetida*, respectively) and average sludge application rates in Europe (i.e. 2 t/ha, EC, 2010) of at least factor 30 (see A.1.3). However, applying assessment factors to extrapolate the results to the ecosystem analogously to the common environmental risk assessment procedure used for chemical substances, factor 100 to 1000 would probably be appropriate, given that only two organisms and only short-term exposure were tested (EC, 2003). As the retrieved safety margins were in the same range (30 to 100), the present test results cannot exclude long-term ecological effects on other organism groups or soil functions from land application of the respective sludges. Comparable studies found that sludge originating from olive-processing or electroplating industry was toxic for plants, collembolans and earthworms, respectively, at realistic application rates, while municipal sludge was found non-toxic (Natal-da-Luz et al., 2009). However, it can be hypothesised that realistic application rates of municipal sludge may have negative effects on plants (Ramirez et al., 2008) in case of applying sludge amendment in soil restoration or for forage crops or pasturelands, where it is often not deeply mixed with the soil.

However, the presence of sludge-associated pollutants in soil does not necessarily manifest in distinct negative effects in the soil ecosystem. A plant growth stimulation effect of sewage sludge is usually observed at low doses as was observed in A.1.2 for growth of *B. napus* and found in similar studies (Petersen et al., 2003, Ramirez et al., 2008). This enhancing effect of nutrient addition can mask negative effects which become evident only when nutrients have been consumed but soil pollution persists (Ramirez et al., 2008), as demonstrated for e.g. triclosan and methyl triclosan accumulation in earthworms (Macherius et al., 2014).

Transferability of wastewater irrigation experiment

The lab-scale experiment of percolating wastewater through soil columns (A.1.4, depicted in Figure 12) was designed to simulate effects from long-term irrigation on the soil and water habitat quality in a condensed time scale of three months.

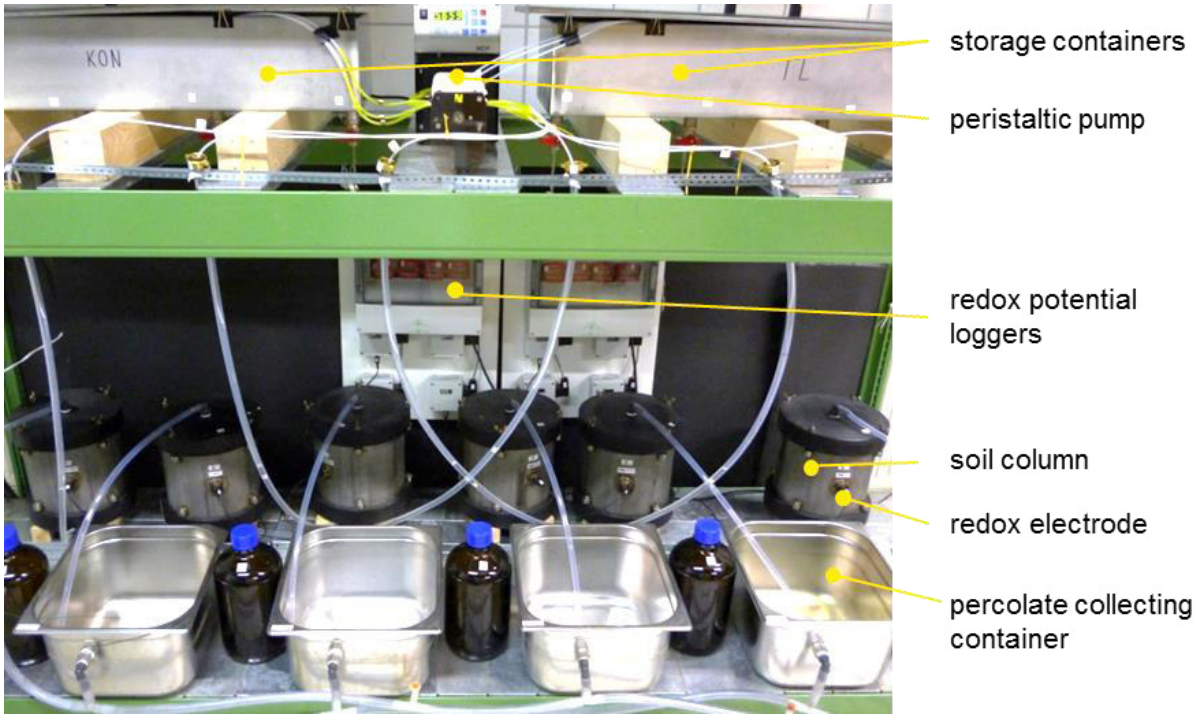


Figure 10: Soil column construction used to percolate four different soils with treated wastewater. (Pre-experiment depicted, using control and test solution as feed with three column replicates each).

Per day, a volume of 10 L percolated a soil surface area of 363 cm^2, corresponding to an irrigation rate of 276 L/m^2*day (equivalent to mm precipitation) and a total irrigation of 21,220 L/m^2 over 77 days experiment duration. This percolation regime was chosen based on the rate applied on experimental field sites of the project ELaN as well as on common percolation rates reported in literature. For long-term wastewater irrigation in agriculture a rate of approximately 500 L/m^2*year is applied on sandy soils at Braunschweig, Germany (Ternes et al., 2007). Likewise, an average of 495 L/m^2*year is used for irrigation in Spain (Munoz et al., 2009). Similarly, treated wastewater was irrigated at a rate of 2 L/m^2*d on a field with reed cultivation in the ELaN project (corresponding to 365 L/m^2*year assuming irrigation during 6 months per year). Compared to this irrigation regime, the total volume of 770 L applied per soil column would simulate 29 years of continuous or 58 years of summer irrigation. As the lab-scale experiment had to condense the time scale, its daily irrigation rate was approximately 140-fold higher than common practise. Compared to the field situation, the removal efficiency of wastewater-borne pollutants may thereby be underestimated because a lower irrigation rate and thus higher residence time of wastewater in soil may increase sorption via

sequestration into soil particles (Dalkmann et al., 2014, Verlicchi and Zambello, 2014). Furthermore, little time was allowed for microbial attack during percolation, for microbial adaptation to take place and influence of plant coverage was excluded (Dalkmann et al., 2014, Li et al., 2014, Verlicchi and Zambello, 2014). However, the intense irrigation may have also overestimated degradability of pollutants because when they occur at trace concentrations, microorganisms usually prefer more easily available carbon sources than pollutants (Verlicchi and Zambello, 2014, Grossberger et al., 2014). Thus, results of the lab-scale experiment cannot predict the exact situation under field conditions but rather indicate trends that are likely to occur.

A shortcoming in the column experiment was the omission of technical replicates, i.e. columns per soil type. Further, the four soils differed not only in general characteristics (e.g. pH, OC, clay content) but also in pre-existing pollutant concentrations. Thus, only one soil, LUFA 2.3 (s4) had no specific anthropogenic influence prior to the experiment, whereas one soil (s1) could be regarded as contaminated site, one (s2) as having moderate and one (s3) having low anthropogenic pollution. However, pollution patterns of old contamination encompassed mostly metals and classical organic pollutants while that of new contamination comprised mainly emerging micropollutants such as PPCPs. Thus, chemical analyses could discriminate between retention of new and leaching of old contaminants. Yet, effects in bioassays were the sum of both interacting processes. For future experiments it would therefore be advisable to apply more column replicates per soil to retrieve a better understanding of the interaction of soil and water properties.

A further limitation was that the retention of pollutants could not be assessed by layers but as average concentration after mixing the whole soil batch of one column of 18 cm length. Yet, wastewater-borne substances adsorb primarily in the first cm of the soil as found for soil passage in bank filtration (Henzler et al., 2014) so that a concentration gradient can be expected in the soil column. This implies that terrestrial organisms living in this top soil layer may be exposed to higher concentrations of wastewater-borne substances under field conditions than those obtained and tested by the present experimental approach.

Choosing an adequate control soil that differs only in the aspect of contamination is important in environmental sample testing because suboptimal performance of test organisms may origin from contamination but also from improper soil properties (Moser and Römbke, 2009). This was taken into account when comparing biological and chemical results per soil, i.e. with and without percolation with treated wastewater. Sample treatment (e.g. sieving, homogenisation, storage, moisture adjustment) of percolated and untreated soils was kept as similar as possible. Therefore, it appears unlikely that other factors than pollution should have caused the observed differences between untreated and percolated soils.

To account for physic-chemical effects from the percolation, i.e. changes in soil structure or trace nutrients, a control percolation with 0.01 mol calcium chloride solution had been conducted in parallel to the soil column percolation with treated wastewater. This cation-rich solution, sometimes called "artificial rainwater", is also used in leaching experiments (Xu et al., 2010, Oppel et al., 2004, OECD 312, 2006b), and was chosen to simulate worst-case mobilisation of metals from the soils (Degryse et al., 2009). As this control treatment gave little additional insight and did not depict a realistic surrogate for irrigation water such as groundwater or surface water, it is not treated in A.1.4. However, the results support the actual findings: The deteriorated habitat function of the soils s4 and s2 as observed from the wastewater percolation was not seen for the rainwater percolation. This supports the conclusion that the habitat deterioration by wastewater resulted from retained pollutants. However, plant growth that was slightly inhibited by wastewater percolation in soil s3 was even more inhibited by rainwater percolation in all soils. This may have been due to the leaching of nutrients from the soil and could suggest that the observed growth inhibition from wastewater was likewise linked to nutrient deficiency (despite fertiliser supply during the plant tests). However, the rainwater percolation may have also increased the soil concentration of calcium and chloride and thus harmed plant growth as sometimes reported for wastewater irrigation (Bedbabis et al., 2014). Due to limited soil analytics, these speculations could not be finally elucidated. Regarding metals, however, a similar or even higher leaching of nickel and aluminium from pre-contaminated soils was seen for the percolation with rainwater compared to that with wastewater. This supports the hypothesis that metals can be mobilised from pre-contaminated soil by wastewater irrigation, but that other, less-buffered irrigation water such as groundwater may lead to the same or even higher leaching.

For future experiments, it would be recommendable to test the alternative scenario to wastewater irrigation by e.g. performing a realistic irrigation water control in parallel. Moreover, sampling and testing of soil at different intervals during wastewater percolation could help defining a timescale at which wastewater irrigation would start causing detrimental effects on soil quality and under what conditions it could be considered safe.

Relevance of findings for land application of treated wastewater

The relevance of observations made in the column percolation experiment (A.1.4) is discussed in the subsequent sections.

Treated wastewater quality

Results of the wastewater percolation experiment demonstrated that the studied effluent exerted phytotoxicity to *L. minor* and *P. subcapitata* despite its compliance with threshold value concentrations regarding priority substances of the Water Framework Directive (WFD, EC 2000).

However, as long as a dilution of 1:16 (i.e. 6.25% sample) causes less than 20% growth inhibition in green algae, the AbwV considers treated wastewater appropriate for discharge (with tighter requirements for effects in fish embryos and *Daphnia*). Thus, only at sampling 3 of the percolation experiment (A.1.4) the treated wastewater may not have met these requirements for a concentration of 89% sample caused 70% inhibition in *P. subcapitata*. While no regulatory threshold values are available for the similarly affected *L. minor*, the undiluted treated wastewater did not affect *D. magna* at any of the three samplings.

Phytotoxicity was likewise observed by Tang et al. (2014) in Australian municipal wastewater using *in-vitro* cell-based bioassays and analysing 299 organic compounds. In an attempt to identify substances responsible for the observed effects, the detected pesticides could sufficiently explain photosynthesis inhibition. This underlines that pesticides and biocides should not be neglected in monitoring municipal wastewater as they are highly efficient against primary producers. Observed cytotoxicity and oxidative stress in treated wastewater, however, could be explained by less than 3% by the considered chemicals (Tang et al., 2014). Better agreement of chemical and biological monitoring was found for the effluent quality of the 45 most-polluting industries in Quebec (e.g. chemical, petrochemical, and metallurgical facilities), where assessment by WET and analysis of Canadian priority chemicals showed that these pollutants could account for overall 43% of observed effects, with greater-than-predicted toxicity for effluents from pulp and paper facilities, and less-than-predicted toxicity for effluents with high metal concentrations (Sarakinos et al., 2000).

Regarding the relevance of micropollutants in treated wastewater, theoretical approaches comparable to the sum TUs method applied in A.1.4 predominantly identified pharmaceuticals to pose an environmental risk: In Greek effluent, a risk to the aquatic environment was predicted for the disinfectant triclosan, the antibiotics trimethroprim and sulfamethoxazole and the analgesic/anti-inflammatory drug diclofenac (Kosma et al., 2014). Likewise, the antibiotics erythromycin and sulfamethoxazole (Gros et al, 2010) and clarithromycin, erythromycin, ofloxacin, sulfamethoxazole and the psychotropic drug fluoxetine (Verlicchi et al., 2012) were assumed to pose an environmental risk in municipal Spanish effluent. For river water that contains diluted effluent, an ecotoxicological risk was estimated e.g. for clarithromycin, sulfamethoxazole and trimethoprim using the sum TUs approach (Valcarcel et al., 2011). An environmental risk was likewise identified for the pharmaceuticals amoxicillin, clarithromycin, erythromycin, sulfamethoxazole and ibuprofen even in the treated effluent that had passed constructed wetlands as a tertiary cleaning step (Verlicchi and Zambello, 2014). It has to be noted, however, that firstly, these studies lack experimental evidence, and secondly, they compare PNECs derived from acute toxicity data and an assessment factor of 1000 and compare it to the highest MEC, thus assuming worst-case scenarios.

To conclude, municipal secondarily treated wastewater may contain pesticides, biocides (i.e. pesticides applied in households) and pharmaceuticals such as antibiotics at concentrations affecting aquatic organisms, predominantly primary producers. Yet, when diluted with surface water, an immediate risk to the receiving aquatic environment should rarely occur.

Effects of soil passage on micropollutants

Soil passage was observed to partly reduce the phytotoxicity of the treated wastewater (A.1.4) and to partially remove many wastewater-borne organic pollutants. Thus, the present results demonstrate that pharmaceuticals which are known to pose an environmental risk to the aquatic habitat such as trimethoprim, clarithromycin, erythromycin and diclofenac (Valcarcel et al., 2011, Kosma et al., 2014, Gros et al., 2010) can be effectively reduced via soil passage and hence improve the chemical quality of the treated wastewater.

However, several pollutants were found to occur unaltered in the percolates after soil passage (A.1.4) which may imply a hazard to groundwater quality. Low removal (≤20%) occurred for e.g. the contrast media diatrizoate and iopamidol, for the antiepileptic carbamazepine, the antibiotic sulfamethoxazole, the antiepileptic primidone, the betablocker sotalol, and the fungicide fluconazole (A.1.4). The first four mentioned substances were likewise detected at µg/L concentrations in groundwater underlying fields irrigated with treated wastewater (Ternes *et al.,* 2007). Thus, the present findings add evidence to the concern that irrigation with treated wastewater can impact groundwater quality. However, a greater threat to groundwater seems to be linked to contamination by point sources, e.g. landfills, intensive and concentrated animal rearing operations, and industrial sites (Postigo and Barcelo, 2015). Apparently, groundwater quality is threatened mostly by water soluble and polar compounds (e.g. polar pesticides, transformation products and artificial sweeteners), but also less water soluble and less polar compounds may be detected (e.g. benzotriazoles, di-ethylhexyl-phthalate, volatile organic compounds and PAHs) when they are comprised in wastewater continuously and at high concentrations (Postigo and Barcelo, 2015).

Demonstrated by the present work, the percolation with treated wastewater can lead to the retention of zinc and a range of wastewater-borne micropollutants in soils (A.1.4). Yet, this does not automatically end in their linear increase and accumulation in soil over time, because soil binding may be reversible and biodegradation and/or uptake into plants may reduce pollutants' soil concentrations (Chefetz et al., 2008, Dalkmann et al., 2014, Grossberger et al., 2014). Diclofenac e.g. was found to be retained in all four wastewater percolated soils, but as anion it should underlie weak and thus easily reversible binding and furthermore seems to be biodegradable under aerobic conditions (Grossberger et al., 2014, Dalkmann et al., 2014). Therefore, it can be hypothesized that diclofenac poses no direct risk to the terrestrial compartment by irrigation with treated wastewater. In

contrast, the neutral substances carbamazepine and benzotriazole, which were found at high concentrations in all percolated soils, can be expected to rather persist in soil due to their low biodegradability (Dalkmann *et al.*, 2014, Grossberger *et al.*, 2014, Reemtsma et al., 2010). Long-term field studies reporting elevated soil concentrations of carbamazepine and other micropollutants such as caffeine and metoprolol support these findings (Dalkmann *et al.*, 2014, Grossberger *et al.*, 2014).

Moreover, long-term wastewater irrigation was found to slow down soil dissipation rates of neutral and cationic organic pollutants, i.e. to reduce their loss over time due to mineralization, transformation, and formation of non-extractable residues (Dalkmann et al., 2014). Interestingly, this could be attributed not to detrimental effects on the microbial community but to changes in soil properties, such as increased organic matter content (Dalkmann et al., 2014).

Soil organic carbon seems to also support the retention of metals from wastewater irrigation as indicated by the elevated zinc concentrations in the peaty compared with the sandy soils after percolation (A.1.4) and reported in literature (Hamilton et al., 2007, Lottermoser 2012, Hoffmann et al., 2002). The role of dissolved (in contrast to particular) organic matter, however, which is introduced via treated wastewater, is difficult to generalize as it may not only increase the co-sorption and cumulative retention of micropollutants but also enhance their mobility and transport in soil, depending on the substance (Chefetz et al., 2008).

Effects of retained micropollutants on organisms

Terrestrial toxicity of micropollutants such as PPCPs is unknown for the majority of candidates so that predicting their effects only from measured soil concentrations is hardly feasible (A.1.4). The question whether or not trace concentrations of organic micropollutants are relevant for effects in soil is thus difficult to answer. Often, effects to terrestrial organisms occur in the mg/kg range, as found for plant growth inhibition by the tested antimycotics and BDDA (A.1.2) or reported for enchytreid toxicity of heavy metals and even antimicrobial and antiparasitic substances (Didden and Römbke, 2001). Yet, terrestrial toxicity can also occur at lower concentrations, underlining the importance of assessing pollutants individually. The parasiticide ivermectin e.g. was found to be highly toxic in the µg/kg range toward terrestrial organisms (56 day NOEC of 2.5 mg/kg d.w. for reproduction of oligochaetes and 54 µg/kg dry dung NOEC for survival development time of dung flies) (Egeler et al., 2010). Using aquatic PNECs and partitioning models in soil, Munoz et al. (2009) identified seven substances, namely erythromycin, gemfibrozil, carbamazepine, ciprofloxacin, diclofenac, sulfamethoxazole and nickel, to result in a hazard to terrestrial organisms from wastewater irrigation. These substances could not be directly linked to the observed effects (A.1.4) but may be relevant as their derived PNECs were indeed exceeded by the soil concentrations after wastewater percolation.

Functions of soil that shall be protected according to the German federal soil protection ordinance (BBSchV, 1999) are: i) food and biomass production; ii) storing, filtering and transformation functions for gases and water; iii) habitat and gene pool diversity; iv) physical and cultural environment for mankind; v) source of raw materials; vi) and the soil habitat function. With the terrestrial and aquatic bioassays applied in the percolation experiment (A.1.4) representing different taxa and trophic levels, the habitat as well as the filtering function of soil was focussed on. Yet, standard bioassays may not be able to detect subtle but important effects for ecosystem health. Behaviour may e.g. be influenced, as demonstrated for the parasiticide imidacloprid affecting earthworms' burrowing behaviour already at low concentrations of 0.2 mg/kg soil d.w. (Dittbrenner et al., 2011). Moreover, standard tests do not cover chronic multi-generation exposure to sublethal concentrations, which may induce increased tolerance levels as found for cadmium toxicity in earthworms (Otomo and Reinecke, 2010) but also increased sensitivity as demonstrated for effects of the fungicide pyrimethanil on *D. magna* (Seeland et al., 2012). Other aspects such as functional endpoints or secondary poisoning of top predators via the terrestrial food chain could not be investigated in the present work. Soil microbial activity at least seems to profit from application of treated wastewater (Filip et al., 1999, del Mar Alguacil et al., 2012).

Overall, it can be concluded from the present work and literature that benefits from reusing treated wastewater for irrigation such as its further purification by soil passage can come at the price of deteriorating soil quality with respect to its habitat suitability for terrestrial organisms as well as its filtering capacity for groundwater protection.

In conclusion, the likely increase of micropollutants in soil from treated wastewater irrigation should be observed with concern when aiming at conserving or not deteriorating soil habitat quality. Therefore, the environmental impacts of treated wastewater irrigation should be assessed by taking into consideration chemical and biological methods and considering the aquatic as well as the terrestrial habitat. Similar approaches have been suggested for the assessment of sediment (Hollert et al., 2002, Kienle et al., 2013), but not yet for wastewater irrigated soils.

2.4. Environmental impact of other deposition routes and land management options

Alternative discharge routes for treated wastewater and sewage sludge as well as environmental consequences of conventional irrigation and fertilisation practices will be scrutinised briefly in the following.

Alternative routes of using treated wastewater and sewage sludge

In countries where the use of treated wastewater for irrigation is accepted, water quality usually has to meet additional requirements beyond those for effluent discharge (Hamilton et al., 2007). In the USA, treated wastewater for irrigation of agricultural fields or golf courses has to comply with drinking water guidelines, while in Australia, 348 organic chemicals are currently monitored and have to comply with health-based guidance values (Asano and Cotruvo, 2004, Hamilton et al., 2007, Tang et al., 2014). If treated wastewater shall be used for aquifer recharge, i.e. indirect potable use, further purification steps are often added after secondary treatment. On the Australian West Coast these steps comprise ultrafiltration, reverse osmosis and disinfection by ultraviolet light, which additionally aim at reducing pathogens (Tang et al., 2014).

Discussing the risks from irrigation of treated wastewater to the aquatic environment, one should scrutinise for comparison the current practice of discharging effluent into surface waters. The rational of protecting groundwater as source for drinking water production is of vital importance; yet, drinking water in Europe is often derived from river bank filtration, i.e. from soil-filtered surface water. This is especially problematic in densely populated urban areas like Berlin with tight water cycles between effluent discharge into surface water and raw water wells along river banks (Massmann et al., 2008, Eggen et al., 2014). Thus, residual contamination of effluent with wastewater-borne pollutants is crucial, irrespective the water or soil discharge route, as short water circles may bring them back to human consumption anyway.

Therefore, optimising the removal efficiency of organic micropollutants by upgrading the conventional WWT with additional, advanced treatment steps has received much attention during the last years (Joss et al., 2008, Margot et al., 2013, Eggen et al., 2014). Mainly two technologies have reached application at real scale: filters with powdered or granulate activated carbon and ozonation. While activated carbon act mainly via adsorption, thus retaining mostly lipophilic and basic substances such as carbamazepine or clarithromycin, ozonation mainly transforms organic contaminants into oxidation products, also affecting polar and acidic substances such as iodinated contrast media or diclofenac (Magdeburg et al., 2014). However, the formation and toxicity of oxidised transformation products has not been well understood yet (Joss et al., 2008). A polishing step such as sand filtration can be added after these advances treatments to support elimination of oxidation products and particle-bound pollutants (Margot et al., 2013, Eggen et al., 2014). Other WWT upgrade technologies comprise membrane application, chorination and irradiation by ultraviolet light, while the latter two presumably have no significant effect on removal of emerging organic pollutants but primarily remove pathogens (Munoz et al., 2009). An implementation of advanced WWT technologies at full-scale has recently been agreed upon for densely populated regions in Switzerland (Eggen et al., 2014).

Simpler and more economic methods for a further purification of secondary effluent (Li et al., 2014) or even replacing conventional WWTPs in small communities can be "constructed wetlands" (Kadlec, 1995). These artificial wetlands with different flow schemes, filling materials and plant coverage (Haberl, 2003, Verlicchi and Zambello, 2014, Dordio and Carvalho, 2013, Anderson et al., 2013) have been shown to effectively reduce suspended solids, biological oxygen demand, phosphorus, nitrogen and metals from treated wastewater (Kadlec, 1995, Haberl et al., 2003). Therefore, they have received increasing interest regarding their potential for micropollutants' removal (Verlicchi and Zambello, 2014, Dordio and Carvalho, 2013, Anderson et al., 2013, Li et al., 2014). Combining different redox zones makes them particularly efficient in the degradation of organic pollutants (Matamoros et al., 2008), because depending on the structure of the individual pollutant either aerobic, denitrifying, or anaerobic conditions are favourable for bacterial attack (Joss et al., 2005). While constructed wetlands in comparison to conventional WWTPs have thus the benefit of being low-cost in construction, operation and maintenance, they have the drawback of being subject to seasonal variation and having high demands for area. For these reasons their application in urban zones is hindered. However, soil filters, i.e. small planted areas allowing for soil passage and subsequent collection and discharge of the percolate, can be applied e.g. to treat occasional raw wastewater overflow from storm water events (Scheurer et al., 2015).

Nutrients contained in sewage sludge, even when not used in agriculture directly, are a valuable resource so that research intensifies on the beneficial recovery of phosphorus, nitrogen, and recently of metals, proteins and enzymes (Lundin et al., 2004, Tyagi and Lo, 2013). Being rich in carbon, sewage sludge is also an important source for energy. Various conventional and advanced sludge treatment technologies are scrutinised for their energy production, e.g. anaerobic digestion of sludge for biogas recovery, co-digestion, incineration and co-incineration with energy recovery, pyrolysis, gasification, supercritical wet oxidation, use in the production of construction materials, and production of biofuels and electricity generation by specific microbes (Fytili and Zabaniotou, 2008, Tyagi and Lo, 2013). However, most processes still possess either economic or environmental drawbacks. Incineration e.g. may cause emissions to air of mercury and mono-nitrogen oxides. Further, for recycling pure phosphorus from sewage sludge ashes, only ashes from the energy demanding mono-incineration can be used and not those from co-incineration of sludge with other wastes (Lundin et al., 2004).

Alternative routes for fertilisation and irrigation

In Germany, an annual amount of about 3 mio t of mineral fertilizer, about 17 mio t of farmyard manure, 2.8 mio t of biowaste and 1.4 mio t of sewage sludge are used in agriculture (UBA, 2001).

The risks for the environment of using sewage sludge and/or treated wastewater should therefore be seen in relation to those from other fertiliser products.

In terms of nutrient leaching to surface and groundwater, other entry routes may pose higher risks than sewage sludge or wastewater application to soil: Phosphorus runoff from field plots having received different types of inorganic and organic fertiliser over a two year period seem to be greater for triple super phosphate and liquid cattle manure than for liquid or dewatered sewage sludge (UBA, 2001). Whereas in sewage sludge, copper, mercury and zinc concentrations are usually problematic for accumulation in soil or leaching to groundwater (Ramirez et al., 2008), organic fertilisers such as manure and pig slurry likewise contain elevated levels of metals, because particularly zinc and copper are applied as food additives to promote animal growth (UBA, 2001). However, contamination of classical organic pollutants such as PAHs and PCBs seem to be considerably higher in sewage sludge compared to other organic fertilisers like pig and cattle slurries and compost (Berset and Holzer, 1995).

Similar to other fertilisers, sewage sludge application in Germany has to safeguard certain threshold values in groundwater and surface water of nitrate and priority pollutants (2006/118/EG, 2000/60/EC). However, the majority of wastewater-borne pollutants is not regulated, considering several hundreds of veterinary pharmaceuticals used in the EU (Kools et al., 2008). While human drugs' concentrations are usually at least partially reduced during WWT, veterinary drugs and their metabolites are often released directly into water via aquaculture and field runoff and to soil via dung and manure from intensively reared animals or animals grazing on pasture (Halling-Sorensen et al., 1998, Boxall et al., 2006, 2012). Prescribed and over the counter veterinary drugs comprise many applications, e.g. anti-infectives and anesthetics in aquaculture, estrogenic drugs for growth enhancement, antibiotics, or tranquilizers in pet animals (Daughton and Ternes, 1999). Especially the exposure of environmental microorganisms to various antibiotics from animal manure can lead to the development of multi-resistant strains that may be transferred back via the food chain (Halling-Sorensen et al., 1998). Overall, a societal understanding of the consequences involved in the use of chemicals and pharmaceuticals is necessary to possibly reduce their usage quantities and incorrect discharge (Eggen et al., 2014). Avoiding or reducing their usage would generally be more economic and efficient than optimising their degradation in WWTPs.

While today water scarcity and the need for irrigation of fields is still an issue in rather few European countries, the need to find innovative solutions to water challenges in the urban, industrial and agriculture contexts is becoming more and more evident also in the North of Europe (COM, 2012). Therefore, a regulatory instrument for water reuse is planned on the European level to be developed in 2015 (JRC, 2014). The experimental percolation of soil columns with treated wastewater presented in A.1.4 could be one approach to assess the potential environmental effects for a specific

soil and effluent. Despite the many water reuse projects that have been carried out in various countries, there are still a number of barriers which prevent the implementation of reusing treated municipal wastewater and greywater not only for agricultural applications but also for urban use like green areas or toilet flushing, recreational use like golf course irrigation, and industrial uses such as washing and cooling. To conclude, there are many areas in water management that have great potential for improving water resource protection in terms of water quantity as well as quality where the reuse of treated wastewater for irrigation in agriculture is only one aspect.

2.5. General conclusions and outlook

The present work helps to improve the understanding of the effects of micropollutants in treated wastewater and digested sewage sludge. Characterising the ecotoxicological hazard and environmental relevance of selected wastewater-borne pollutants, three antimycotics and one cationic surfactant, demonstrated their high toxicity toward primary producers. Moreover, it was found that ecotoxicity of sludge-associated organic pollutants can be adequately assessed by standard plant tests, i.e. without the need to amend test soil with sewage sludge for a realistic exposure scenario. The screening of digested sludges from different advanced treatment technologies for terrestrial ecotoxicity indicated that stabilisation indeed improved their habitat quality and that none of the samples posed an immediate risk to soil organisms. The soil column percolation approach developed and employed in the present work represents a feasible tool to assess at lab-scale the integrated impact on the aquatic as well as the terrestrial compartment of prolonged soil irrigation with treated wastewater. Results can be used to point at potential effects such as leaching of wastewater-born pollutants to groundwater and pollutant accumulation in soil before applications at field-scale and thus support risk management decisions.

Using treated wastewater for the irrigation of soil should be assessed on a case-by-case basis concluding from the present work. While soil passage can remove wastewater-borne pollutants from the treated wastewater, this may be due to their adsorption to soil, consequently transferring the risk from one compartment to the other. If a further purification of effluent is the objective for wastewater irrigation, more efficient methods should be chosen such as constructed wetlands or soil filters that expose a limited area of non-arable soil and collect the percolate and lead it into surface waters.

Findings of the present work on the ecotoxicological properties of sewage sludge from novel stabilisation technologies support the development of future research directions for the treatment and management of sewage sludge. Regarding the efficiency of wastewater treatment it may therefore be reasonable to optimise sludge quality in one WWTP, where the sludge could be used for agriculture, but optimise removal of pollutants from the effluent in another, where the reuse of treated wastewater for irrigation is an option. A compromise in the agricultural usage of sewage sludge may be the

restriction to sludge of a certain origin, e.g. from advanced WWTPs, and to sites with already established anthropogenic impact. Meanwhile, the technical recovery of nutrients from sewage sludge, mainly phosphorus, will have to be promoted against the background of declining natural phosphorus resources as already reflected in recent European research initiatives.

In the near future, complete degradation of wastewater-borne pollutants by conventional WWTPs will hardly be feasible while new substances will continuously be developed and introduced into the sewers. As a consequence, the use of treated wastewater for irrigation or aquifer recharge and the land application of sewage sludge will contribute to some extend to spread contaminants in the environment. Notwithstanding, recycling of treated wastewater remains an important water resource in arid and semi-arid areas, and land application in agriculture of sewage sludge is a sensible approach for recycling nutrients such as phosphorus and organic matter. Avoiding and reducing the application of poorly degradable chemicals and improving their degradation during WWT will therefore be an ongoing challenge to face by modern society. In general, optimising the level of degradation during WWT should be accompanied by a reduction of pollutant input into sewers at the source, e.g. at hospitals and households, being more economic as well as efficient.

On the European level, an increasing interest in using treated wastewater for agricultural irrigation is reflected in research projects at present but will have to be accompanied by the development of certain standards. In agricultural areas where irrigation is needed, however, municipal WWTPs usually discharge low effluent volumes so that they can only substitute parts of the irrigation water. Moreover, nutrients concentrations in treated wastewater are not sufficient to completely substitute fertiliser. As the present work underlines, wastewater irrigation on soil should not be a general, ubiquitously applicable solution. A future scenario could therefore be the permission of reusing low-polluted treated wastewater in certain constrained applications and only on soils in regions with water scarcity and no direct contact with groundwater. The here presented soil column irrigation approach may help to assess potential detrimental effects on the soil and water habitat quality on a case-by-case basis and support the selection of adequate wastewater and soil.

3. References

AbwV (Abwasserverordnung) in der Fassung der Bekanntmachung vom 17. Juni 2004 (BGBl. I S. 1108, 2625), die zuletzt durch Artikel 1 der Verordnung vom 2. September 2014 (BGBl. I S. 1474) geändert worden ist.

AbfKlärV (Klärschlammverordnung) vom 15. April 1992 (BGBl. I S. 912), zuletzt geändert durch Artikel 5 Absatz 12 des Gesetzes vom 24. Februar 2012 (BGBl. I S. 212).

Allen H, Hall R, Brisbin T (1980). Metal Speciation - Effects on Aquatic Toxicity. Environ Sci Technol 14:441–443.

Altenburger R, Krüger J, Eisenträger A (2010). Proposing a pH stabilised nutrient medium for algal growth bioassays. Chemosphere 78:864–870.

Anderson JC, Carlson JC, Low JE, Challis JK, Wong CS, Knapp CW, Hanson ML (2013). Performance of a constructed wetland in Grand Marais, Manitoba, Canada: Removal of nutrients, pharmaceuticals, and antibiotic resistance genes from municipal wastewater. Chemistry Central Journal 7:54.

Angelakis AN, Marecos Do Monte MHF, Bontoux L, Asano T (1999). The status of wastewater reuse practice in the Mediterranean basin: Need for guidelines. Water Research 33:2201–2217.

Aruoja V, Kurvet I, Dubourguier HC, Kahru A (2004). Toxicity testing of heavy-metal-polluted soils with algae *Selenastrum capricornutum*: a soil suspension assay. Environ Toxicol 19:396–402.

Asano T, Cotruvo JA (2004). Groundwater recharge with reclaimed municipal wastewater: Health and regulatory considerations. Water Research 38:1941–1951.

Backhaus T, Porsbring T, Arrhenius A, Brosche S, Johansson P, Blanck H (2011). Single-substance and mixture toxicity of five pharmaceuticals and personal care products to marine periphyton communities. Environ Toxicol Chem 30:2030–2040.

Balla D, Maassen S, Richter E, Guimaraes B, Coors A. Dissipation of micropollutants in rewetted fen peatlands - a field study with treated wastewater usage. *in preparation*

Barron L, Tobin J, Paull B (2008). Multi-residue determination of pharmaceuticals in sludge and sludge enriched soils using pressurized liquid extraction, solid phase extraction and liquid chromatography with tandem mass spectrometry. Journal of Environmental Monitoring 10:353.

BBodSchV (Bundesbodenschutzverordnung - Federal Ordinance on Soil Protection and Contaminated Sites) of 12 July 1999 (Federal Law Gazette I, no. 36, p. 1554); most recently amended on 24 February 2012. Federal Law Gazette (BGBl.) I, p. 212.

BBodSchG (Bundesbodenschutzgesetz - Federal Soil Protection Act) of 17 March 1998 (Federal Law Gazette (BGBl.) I, p. 502, most recently amended on 24 February 2012 by Article 5 (30) of the Act Reorganizing the Closed Substance Cycle and Waste Management Act, Federal Law Gazette I page 212.

Bedbabis S, Rouina B Ben, Boukhris M, Ferrara G (2014). Effect of irrigation with treated wastewater on soil chemical properties and infiltration rate. Journal of Environmental Management 133:45–50.

Berset JD, Holzer R (1995). Organic micropollutants in Swiss agriculture: Distribution of polynuclear aromatic hydrocarbons (PAH) and polychlorinated biphenyls (PCB) in soil, liquid manure, sewage sludge and compost samples; a comparative study. International Journal of Environmental Analytical Chemistry 59:145–165.

Beyer J, Petersen K, Song Y, Ruus A, Grung M, Bakke T, Tollefsen KE (2014). Environmental risk assessment of combined effects in aquatic ecotoxicology: A discussion paper. Marine Environmental Research 96:81–91.

Bîrsan E., Luca C., 2010. Speciation of heavy metals in surface waters polluted by anthropogenic activities. Scientific Study & Research, Chemistry & Chemical Engineering, Biotechnology, Food Industry 11 (4):439 – 448.

Borgman O, Chefetz B (2013). Combined effects of biosolids application and irrigation with reclaimed wastewater on transport of pharmaceutical compounds in arable soils. Water Research 47:3431–3443.

Botti P, Virdis A, Solinas G, Buscarinu P, Ferralis M, Marras G, Spanu P, Vacca S (2009). Reclamation and agricultural reuse of wastewater: the experience of the Cagliari sewage treatment plant (Sardinia, Italy). Water Science and Technology 59:65–72.

Boxall ABA, Johnson P, Smith EJ, Sinclair CJ, Stutt E, Levy LS (2006). Uptake of veterinary medicines from soils into plants. Journal of Agricultural and Food Chemistry 54:2288–2297.

Boxall ABA, Rudd MA, Brooks BW, Caldwell DJ, Choi K, Hickmann S, Innes E, Ostapyk K, Staveley JP, Verslycke T, Ankley GT, Beazley KF, Belanger SE, Berninger JP, Carriquiriborde P, Coors A, Deleo PC, Dyer SD, Ericson JF, Gagne F, Giesy JP, Gouin T, Hallstrom L, Karlsson MV, Larsson DGJ, Lazorchak JM, Mastrocco F, McLaughlin A, McMaster ME, Meyerhoff RD, Moore R, Parrott JL, Snape JR, Murray-Smith R, Servos MR, Sibley PK, Straub JO, Szabo ND, Topp E, Tetreault GR, Trudeau VL, Der Kraak G Van (2012). Pharmaceuticals and personal care products in the environment: What are the big questions? Environ Health Perspect 120:1221–1229.

Braguglia CM, Coors A, Gallipoli A, Gianico A, Guillon E, Kunkel U, Mascolo G, Richter E, Ternes TA, Tomei MC, Mininni G (2014). Quality assessment of digested sludges produced by advanced stabilization processes. Environ Sci Pollut Res:1–20.

Briggs GG, Bromilow RH, Evans AA (1982). Relationships between lipophilicity and root uptake and translocation of non-ionised chemicals by barley. Pesticide Science 13:495–504.

Carbonell G, Pro J, Gómez N, Babín MM, Fernández C, Alonso E, Tarazona JV (2009). Sewage sludge applied to agricultural soil: Ecotoxicological effects on representative soil organisms. Ecotoxicology and Environmental Safety 72:1309–1319.

Casado J, Rodríguez I, Ramil M, Cela R (2014). Selective determination of antimycotic drugs in environmental water samples by mixed-mode solid-phase extraction and liquid chromatography quadrupole time-of-flight mass spectrometry. Journal of Chromatography A 1339:42–49.

Cedergreen N (2014). Quantifying synergy: A systematic review of mixture toxicity studies within environmental toxicology (A Nazir, Ed.). PLoS ONE 9:e96580.

CEN (2005). Characterization of waste — preparation of waste samples for ecotoxicity tests. EN 14735, Brussels, Belgium.

CEN (2011). Characterization of waste guidance on the use of ecotoxicity tests applied to waste CEN/TR 16110, Brussels, Belgium.

Chapman PM (2000). Whole effluent toxicity testing - Usefulness, level of protection, and risk assessment. Environ Toxicol Chem 19:3–13.

Chefetz B, Mualem T, Ben-Ari J (2008). Sorption and mobility of pharmaceutical compounds in soil irrigated with reclaimed wastewater. Chemosphere 73:1335–1343.

Chen H, Li X, Zhu S (2012). Occurrence and distribution of selected pharmaceuticals and personal care products in aquatic environments: a comparative study of regions in China with different urbanization levels. Environ Sci Pollut Res 19:2381–2389.

Chen Z-F, Ying G-G, Ma Y-B, Lai H-J, Chen F, Pan C-G (2013). Typical azole biocides in biosolid-amended soils and plants following biosolid applications. J Agric Food Chem 61:6198–6206.

Chen Y, Geurts M, Sjollema SB, Kramer NI, Hermens JLM, Droge STJ (2014). Acute toxicity of the cationic surfactant C12-benzalkonium in different bioassays: How test design affects bioavailability and effect concentrations: Acute toxicity of the cationic surfactant C12-benzalkonium. Environmental Toxicology and Chemistry 33:606–615

Clara M, Scharf S, Scheffknecht C, Gans O (2007). Occurrence of selected surfactants in untreated and treated sewage. Water Res 41:4339–4348.

Clarke BO, Smith SR (2011). Review of "emerging" organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids. Environment International 37:226–247.

COM (Commission of the European Communities) (2001). White Paper - Strategy for a future Chemicals Policy, COM (2001) 88 final, Brussels, Belgium.

COM (Commission of the European Communities) (2012). Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions. A Blueprint to Safeguard Europe's Water Resources, COM (2012) 673 final, Brussels, Belgium.

Coors A, Hammers-Wirtz M, Ratte HT (2004). Adaptation to environmental stress in *Daphnia magna* simultaneously exposed to a xenobiotic. Chemosphere 56:395–404.

Coors A, Weisbrod B, Schoknecht U, Sacher F, Kehrer A (2014). Predicting acute and chronic effects of wood preservative products in *Daphnia magna* and *Pseudokirchneriella subcapitata* based on the concept of concentration addition. Environ Toxicol Chem 33:382–393.

Cordy GE, Duran NL, Bouwer H, Rice RC, Furlong ET, Zaugg SD, Meyer MT, Barber LB, Kolpin DW (2004). Do pharmaceuticals, pathogens, and other organic waste water compounds persist when waste water is used for recharge? Ground Water Monitoring & Remediation 24:58–69.

Corrêa J, Reichman C, Salgado HRN, Vianna-Soares CD (2012). Performance characteristics of high performance liquid chromatography first order derivative UV spectrophotometry and bioassay for fluconazole determination in capsules. Quim. Nova 35 (3):530-534.

Crane M, Watts C, Boucard T (2006). Chronic aquatic environmental risks from exposure to human pharmaceuticals. Sci Total Environ 367:23–41.

Dalkmann P, Broszat M, Siebe C, Willaschek E, Sakinc T, Huebner J, Amelung W, Grohmann E, Siemens J (2012). Accumulation of pharmaceuticals, enterococcus, and resistance genes in soils irrigated with wastewater for zero to 100 years in Central Mexico. PLoS ONE 7:e45397.

Dalkmann P, Siebe C, Amelung W, Schloter M, Siemens J (2014). Does long-term irrigation with untreated wastewater accelerate the dissipation of pharmaceuticals in soil? Environ Sci Technol 48:4963–4970.

Daughton CG (2004). Non-regulated water contaminants: emerging research. Environmental Impact Assessment Review 24:711–732.

Daughton CG, Ternes TA (1999). Pharmaceuticals and personal care products in the environment: Agents of subtle change? Environ Health Perspect 107:907–938.

de Vlaming V, Connor V, DiGiorgio C, Bailey HC, Deanovic LA, Hinton DE (2000). Application of whole effluent toxicity test procedures to ambient water quality assessment. Environmental Toxicology and Chemistry 19:42–62.

del Mar Alguacil M, Torrecillas E, Torres P, García-Orenes F, Roldán A (2012). Long-term effects of irrigation with waste water on soil AM fungi diversity and microbial activities: The implications for agro-ecosystem resilience. PLoS ONE 7:e47680.

Degryse F, Smolders E, Parker DR (2009). Partitioning of metals (Cd, Co, Cu, Ni, Pb, Zn) in soils: concepts, methodologies, prediction and applications – a review. European Journal of Soil Science 60:590–612.

Didden W, Römbke J (2001). Enchytraeids as indicator organisms for chemical stress in terrestrial ecosystems. Ecotoxicology and Environmental Safety 50:25–43.

Directive 86/278/EEC (Sewage Sludge Directive), Council Directive of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture. OJ L 181, 4.7.1986, p. 6–12.

Directive 91/689/EEC of 12 December 1991 on hazardous waste. OJ L 377 , 31/12/1991 P. 0020 – 0027.

Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council. OJ L 348/84.

Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. OJ L 226/1.

Dittbrenner N, Moser I, Triebskorn R, Capowiez Y (2011). Assessment of short and long-term effects of imidacloprid on the burrowing behaviour of two earthworm species (*Aporrectodea caliginosa* and *Lumbricus terrestris*) by using 2D and 3D post-exposure techniques. Chemosphere 84:1349–1355.

Dordio AV, Carvalho AJP (2013). Organic xenobiotics removal in constructed wetlands, with emphasis on the importance of the support matrix. Journal of Hazardous Materials 252–253:272–292.

Dorta-Santos M, Tejedor M, Jiménez C, Hernández-Moreno J, Palacios-Díaz M, Díaz F (2014). Recycled urban wastewater for irrigation of *Jatropha curcas* L. in abandoned agricultural arid land. Sustainability 6:6902–6924.

DüMV (Düngemittelverordnung) vom 5. Dezember 2012 (BGBl. I S. 2482), die durch Artikel 1 der Verordnung vom 27. Mai 2015 (BGBl. I S. 886) geändert worden ist.

EC (European Commission) (2003). Technical Guidance Document in support of Commission Directive 93/67/EEC on risk assessment for new notified substances and Commission Regulation (EC) No 1488/94 on risk assessment for existing substances, Part II. Brussels, Belgium.

European Waste Catalogue, 2000/532/EC: Commission Decision of 3 May 2000 replacing Decision 94/3/EC establishing a list of wastes pursuant to Article 1(a) of Council Directive 75/442/EEC on waste and Council Decision 94/904/EC establishing a list of hazardous waste pursuant to Article 1(4) of Council Directive 91/689/EEC on hazardous waste (notified under document number C(2000) 1147) (Text with EEA relevance) OJ L 226, 6.9.2000, p. 3–24.

Commission Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. OJ L 167.

Commission Regulation (EU) No 900/2014 of 15 July 2014 amending, for the purpose of its adaptation to technical progress, Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). OJ L 247/1

Egeler P, Gilberg D, Fink G, Duis K (2010). Chronic toxicity of ivermectin to the benthic invertebrates *Chironomus riparius* and *Lumbriculus variegatus*. J Soils Sediments 10:368–376.

Eggen RIL, Hollender J, Joss A, Schärer M, Stamm C (2014). Reducing the discharge of micropollutants in the aquatic environment: The benefits of upgrading wastewater treatment plants. Environmental Science and Technology 48:7683–7689.

EMA (European Medicines Agency) (2012). EMA/888361/2011 assessment report on diflucan (fluconazole), London, UK. [http://www.ema.europa.eu/docs/en_GB /document_library/Referrals_document/Diflucan_30/WC500121953.pdf, accessed 26 February 2015].

EMA (European Medicines Agency) (2014). EMA/CHMP/534845/2014 assessment report on ketoconazole, London, UK, [http://www.ema.europa.eu/docs/en_GB/document_library/ EPAR_-_Public_assessment_ report/human/003906/WC500181493.pdf, accessed 26 February 2015].

EMEA (European Medicines Agency) (2006). Guideline on the environmental risk assessment of medicinal products for human use. June 2006; Doc. Ref. EMEA/CHMP/SWP/4447/00.

EMEA (European Medicines Agency) (2008). Guideline on environmental impact assessment for veterinary medicinal products in support of the VICH guidelines GL6 (Phase I) and GL38 (Phase II). November 2008; Ref. No. EMEA/CVMP/ERA/418282/ 2005-Rev.1.

Erickson RJ (2013). The biotic ligand model approach for addressing effects of exposure water chemistry on aquatic toxicity of metals: Genesis and challenges. Environ Toxicol Chem 32:1212–1214.

Eriksson E, Christensen N, Ejbye Schmidt J, Ledin A (2008). Potential priority pollutants in sewage sludge. Desalination 226:371–388.

Escher BI, Allinson M, Altenburger R, Bain PA, Balaguer P, Busch W, Crago J, Denslow ND, Dopp E, Hilscherova K, Humpage AR, Kumar A, Grimaldi M, Jayasinghe BS, Jarosova B, Jia A, Makarov S, Maruya KA, Medvedev A, Mehinto AC, Mendez JE, Poulsen A, Prochazka E, Richard J, Schifferli A, Schlenk D, Scholz S, Shiraishi F, Snyder S, Su G, Tang JYM, Burg B van der, Linden SC van der, Werner I, Westerheide SD, Wong CKC, Yang M, Yeung BHY, Zhang X, Leusch FDL (2014). Benchmarking organic micropollutants in wastewater, recycled water and drinking water with *in vitro* bioassays. Environmental Science and Technology 48:1940–1956

Escher BI, Bramaz N, Richter M, Lienert J (2006). Comparative ecotoxicological hazard assessment of beta-blockers and their human metabolites using a mode-of-action-based test battery and a QSAR approach. Environmental Science and Technology 40:7402–7408.

Fatta-Kassinos D, Kalavrouziotis IK, Koukoulakis PH, Vasquez MI (2011). The risks associated with wastewater reuse and xenobiotics in the agroecological environment. Sci Total Environ 409:3555–3563.

Feiler U, Höss S, Ahlf W, Gilberg D, Hammers-Wirtz M, Hollert H, Meller M, Neumann-Hensel H, Ottermanns R, Seiler T-B, Spira D, Heininger P (2013). Sediment contact tests as a tool for the assessment of sediment quality in German waters. Environmental Toxicology and Chemistry 32:144–155.

Filip Z, Kanazawa S, Berthelin J (1999). Characterization of effects of a long-term wastewater irrigation on soil quality by microbiological and biochemical parameters. Z Pflanzenernähr Bodenk 162:409–413.

Florence TM (1982). The speciation of trace elements in waters. Talanta 29:345–364.

Fytili D, Zabaniotou A (2008). Utilization of sewage sludge in EU application of old and new methods — A review. Renewable and Sustainable Energy Reviews 12:116–140.

Garcia-Valcalrcel AI, Tadeo JL (2011). Determination of azoles in sewage sludge from Spanish wastewater treatment plants by liquid chromatography-tandem mass spectrometry. JSepScience 34:1228–1235.

Gerba CP (2015). Quaternary ammonium biocides: efficacy in application. Applied and Environmental Microbiology 81:464–469.

Golsteijn L, Iqbal MS, Cassani S, Hendriks HWM, Kovarich S, Papa E, Rorije E, Sahlin U, Huijbregts MAJ (2014). Assessing predictive uncertainty in comparative toxicity potentials of triazoles. Environmental Toxicology and Chemistry 33:293–301.

Graber ER, Gerstl Z (2011). Organic micro-contaminant sorption, transport, accumulation, and root uptake in the soil-plant continuum as a result of irrigation with treated wastewater. Isr J Plant Sci 59:105–114.

Gros M, Petrović M, Ginebreda A, Barceló D (2010). Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes. Environment International 36:15–26.

Grossberger A, Hadar Y, Borch T, Chefetz B (2014). Biodegradability of pharmaceutical compounds in agricultural soils irrigated with treated wastewater. Environ Pollut 185:168–177.

GrwV (Grundwasserverordnung) Ordinance on the Protection of Groundwater (Groundwater Ordinance) of 9 November 2010, Federal Law Gazette (BGBl.) I, p. 1513.

Haberl RSG (2003). Constructed wetlands for the treatment of organic pollutants. Journal of Soils and Sediments 3:109–124.

Halling-Sørensen B, Nors Nielsen S, Lanzky PF, Ingerslev F, Holten Lützhøft HC, Jørgensen SE (1998). Occurrence, fate and effects of pharmaceutical substances in the environment- A review. Chemosphere 36:357–393.

Hamilton AJ, Stagnitti F, Xiong X, Kreidl SL, Benke KK, Maher P (2007). Wastewater irrigation: The state of play. Vadose Zone J 6:823–840.

Harrison EZ, Oakes SR, Hysell M, Hay A (2006). Organic chemicals in sewage sludges. Sci Total Environ 367:481–497.

Hassold E, Backhaus T (2009). Chronic toxicity of five structurally diverse demethylase-inhibiting fungicides to the crustacean *Daphnia magna*: A comparative assessment. EnvironToxicolChem 28:1218–1226.

Henneberg A, Bender K, Blaha L, Giebner S, Kuch B, Köhler H-R, Maier D, Oehlmann J, Richter D, Scheurer M, Schulte-Oehlmann U, Sieratowicz A, Ziebart S, Triebskorn R (2014). Are in vitro methods for the detection of endocrine potentials in the aquatic environment predictive for in vivo effects? Outcomes of the projects SchussenAktiv and SchussenAktivplus in the Lake Constance area, Germany (JA Craft, Ed.). PLoS ONE 9:e98307.

Henzler AF, Greskowiak J, Massmann G (2014). Modeling the fate of organic micropollutants during river bank filtration (Berlin, Germany). Journal of Contaminant Hydrology 156:78–92.

Hernando MD, Mezcua M, Fernández-Alba AR, Barceló D (2006). Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. Talanta 69:334–342.

Höss S, Ahlf W, Fahnenstich C, Gilberg D, Hollert H, Melbye K, Meller M, Hammers-Wirtz M, Heininger P, Neumann-Hensel H, Ottermanns R, Ratte H-T, Seiler T-B, Spira D, Weber J, Feiler U (2010). Variability of sediment-contact tests in freshwater sediments with low-level anthropogenic contamination – Determination of toxicity thresholds. Environmental Pollution 158:2999–3010

Hof A (2008). Is there a serious risk of resistance development to azoles among fungi due to the widespread use and long-term application of azole antifungals in medicine? Drug Resistance Updates 11: 25–31.

Hoffmann C, Savric I, Jozefaciuk G, Hajnos M, Sokołowska Z, Renger M, Marschner B (2002). Reaction of sewage farm soils to different irrigation solutions in a column experiment 2. Heavy metals and their leaching. Z Pflanzenernähr Bodenk 165:67–71.

Hollert N, Dürr M, Olsman H, Halldin K, Bavel E van, Brack W, Tysklind M, Engwall M, Braunbeck T (2002). Biological and chemical determination of dioxin-like compounds in sediments by means of a sediment triad approach in the catchment area of the river Neckar. Ecotoxicology 11:323–336.

Hund-Rinke K, Simon M (2004). Terrestrial ecotoxicity of eight chemicals in a systematic approach (7 pp). J Soils Sediments 5:59–65.

Hund-Rinke K, Wiechering H (2001). Earthworm avoidance test for soil assessments. J Soils and Sediments 1:15–20.

Hund-Rinke K (2005). Experiences with novel approaches in earthworm testing alternatives (7 pp). Journal of Soils and Sediments 5:233–239.

Ilias A, Panoras A, Angelakis A (2014). Wastewater recycling in Greece: The case of Thessaloniki. Sustainability 6:2876–2892.

ISO (International Organization for Standardization) (2003). Soil quality — Guidance on the ecotoxicological characterization of soils and soil materials. ISO 15799, Geneve, Switzerland.

ISO (International Organization for Standardization) (2008): Soil quality – Guidance on the assessment of tests applied in the field of ecotoxicological characterization of soils and soil materials. ISO 17616. Geneve, Switzerland.

ISO (International Organization for Standardization) (2010). Soil Quality - Avoidance test for evaluating the quality of soils and the toxicity of chemicals. Test with earthworms (*Eisenia fetida/andrei*). ISO 17512-1. Geneve, Switzerland.

Janer G, Lyssimachou A, Bachmann J, Oehlmann J, Schulte-Oehlmann U, Porte C (2006). Sexual dimorphism in esterified steroid levels in the gastropod *Marisa cornuarietis*: the effect of xenoandrogenic compounds. Steroids 71:435–444.

Jänsch S, Amorim MJ, Römbke J (2005). Identification of the ecological requirements of important terrestrial ecotoxicological test species. Environ Rev 13:51–83.

Joss A, Keller E, Alder AC, Göbel A, McArdell CS, Ternes T, Siegrist H (2005). Removal of pharmaceuticals and fragrances in biological wastewater treatment. Water Research 39:3139–3152.

Joss A, Siegrist H, Ternes TA (2008). Are we about to upgrade wastewater treatment for removing organic micropollutants? Water Science and Technology 57:251.

JRC (Joint Research Center of the European Commission) (2014). Water Reuse in Europe - Relevant guidelines, needs for and barriers to innovation. ISBN: 978-92-79-44399-2.

Kadlec RH (1995). Overview: Surface flow constructed wetlands. Water Science and Technology 32:1–12.

Kah M, Brown CD (2007). Prediction of the adsorption of ionizable pesticides in soils. J Agric Food Chem 55:2312–2322.

Kahle M, Buerge IJ, Hauser A, Müller MD, Poiger T (2008). Azole fungicides: occurrence and fate in wastewater and surface waters. Environmental Science and Technology 42:7193–7200.

Kienle C, Langer-Jaesrich M, Baumberger D, Hohmann D, Santiago S, Köhler H-R, Zürrer D, Gerhardt A (2013). Integrated toxicity evaluation of a pulp deposit using organisms of different trophic levels. Journal of Soils and Sediments 13:1611–1625.

Kiseleva A, Tarachovskaya E, Shishova M (2012). Biosynthesis of phytohormones in algae. Russian Journal of Plant Physiology 59:595–610.

Köck-Schulmeyer M, Villagrasa M, López de Alda M, Céspedes-Sánchez R, Ventura F, Barceló D (2013). Occurrence and behavior of pesticides in wastewater treatment plants and their environmental impact. Science of The Total Environment 458–460:466–476.

Kools SAE, Boxall A, Moltmann JF, Bryning G, Koschorreck J, Knacker T (2008). A ranking of European veterinary medicines based on environmental risks. Integr Environ Assess Manag 4:399–408.

Kosma CI, Lambropoulou DA, Albanis TA (2014). Investigation of PPCPs in wastewater treatment plants in Greece: Occurrence, removal and environmental risk assessment. Science of The Total Environment 466-467:421–438.

Kreuzinger N, Fuerhacker M, Scharf S, Uhl M, Gans O, Grillitsch B (2007). Methodological approach towards the environmental significance of uncharacterized substances — quaternary ammonium compounds as an example. Desalination 215:209–222.

Krauss M, Singer H, Hollender J (2010). LC–high resolution MS in environmental analysis: from target screening to the identification of unknowns. Analytical and Bioanalytical Chemistry 397:943–951

Kümmerer K, Alexy R, Hüttig J, Schöll A (2004). Standardized tests fail to assess the effects of antibiotics on environmental bacteria. Water Res 38:2111–2116.

Lamb D, Kelly D, Kelly S (1999). Molecular aspects of azole antifungal action and resistance. Drug Resistance Updates 2:390–402.

Lamb D, Cannieux M, Warrilow AGS, Bak S, Kahn RA, Manning NJ, Kelly DE, Kelly SL (2001). Plant sterol 14 alpha-demethylase affinity for azole fungicides. Biochemical and Biophysical Research Communications 284:845–849

Langdon KA, Warne MSJ, Kookana R.S. (2010). Aquatic hazard assessment for pharmaceuticals, personal care products and endocrine-disrupting compounds form biosolids-amended land. Integr Environ Assess Manag 6:663–676.

Leusch FDL, Khan SJ, Gagnon MM, Quayle P, Trinh T, Coleman H, Rawson C, Chapman HF, Blair P, Nice H, Reitsema T (2014). Assessment of wastewater and recycled water quality: A comparison of lines of evidence from *in vitro*, *in vivo* and chemical analyses. Water Research 50:420–431

Li Y, Zhu G, Ng WJ, Tan SK (2014). A review on removing pharmaceutical contaminants from wastewater by constructed wetlands: Design, performance and mechanism. Science of The Total Environment 468–469:908–932.

Lindberg RH, Fick J, Tysklind M (2010). Screening of antimycotics in Swedish sewage treatment plants - Waters and sludge. Water Research 44:649–657.

Lischeid G, Nathkin M (2011). The potential of land-use change to mitigate water scarcity in Northeast Germany – a review. DIE ERDE – Journal of the Geographical Society of Berlin 142:97–113.

Lottermoser BG (2012). Effect of long-term irrigation with sewage effluent on the metal content of soils, Berlin, Germany. Environ Geochem Health 34:67–76.

Lundin M, Olofsson M, Pettersson G., Zetterlund H (2004). Environmental and economic assessment of sewage sludge handling options. Resources, Conservation and Recycling 41:255–278.

Macherius A, Lapen DR, Reemtsma T, Römbke J, Topp E, Coors A (2014). Triclocarban, triclosan and its transformation product methyl triclosan in native earthworm species four years after a commercial-scale biosolids application. Science of The Total Environment 472:235–238.

Macikova P, Groh KJ, Ammann AA, Schirmer K, Suter MJ-F (2014). Endocrine disrupting compounds affecting corticosteroid signaling pathways in Czech and Swiss Waters: Potential Impact on Fish. Environmental Science and Technology 48:12902–12911.

Magdeburg A, Stalter D, Schlüsener M, Ternes T, Oehlmann J (2014). Evaluating the efficiency of advanced wastewater treatment: Target analysis of organic contaminants and (geno-) toxicity assessment tell a different story. Water Research 50:35–47.

Malchi T, Maor Y, Tadmor G, Shenker M, Chefetz B (2014). Irrigation of root vegetables with treated wastewater: evaluating uptake of pharmaceuticals and the associated human health risks. Environmental Science and Technology 48:9325–9333

Maltby L, Brock TCM, Brink PJ Van den (2009). Fungicide Risk Assessment for Aquatic Ecosystems: Importance of Interspecific Variation, Toxic Mode of Action, and Exposure Regime. Environmental Science and Technology 43:7556–7563.

Maltby L, Clayton SA, Yu H, McLoughlin N, Wood RM, Yin D (2000). Using single-species toxicity tests, community-level responses, and toxicity identification evaluations to investigate effluent impacts. EnvironToxicolChem 19:151–157

Mantis I, Voutsa D, Samara C (2005). Assessment of the environmental hazard from municipal and industrial wastewater treatment sludge by employing chemical and biological methods. Ecotoxicol Environ Saf 62:397–407.

Margot J, Kienle C, Magnet A, Weil M, Rossi L, Alencastro LF de, Abegglen C, Thonney D, Chevre N, Schaerer M, Barry DA (2013). Treatment of micropollutants in municipal wastewater: Ozone or powdered activated carbon? Sci Total Environ 461:480–498

Martínez-Carballo E, Sitka A, González-Barreiro C, Kreuzinger N, Fürhacker M, Scharf S, Gans O (2007). Determination of selected quaternary ammonium compounds by liquid chromatography with mass spectrometry. Part I. Application to surface, waste and indirect discharge water samples in Austria. Environmental Pollution 145:489–496.

Massmann G, Dünnbier U, Heberer T, Taute T (2008). Behaviour and redox sensitivity of pharmaceutical residues during bank filtration - Investigation of residues of phenazone-type analgesics. Chemosphere 71:1476–1485.

Matamoros V, Caselles-Osorio A, Garc_i_a J, Bayona JM (2008). Behaviour of pharmaceutical products and biodegradation intermediates in horizontal subsurface flow constructed wetland. A microcosm experiment. SciTotal Environ 394:171–176.

Matthiessen P, Weltje L (2015). A review of the effects of azole compounds in fish and their possible involvement in masculinization of wild fish populations. Critical Reviews in Toxicology 45:453–467

Moser H, Römbke J (2009). The ecotoxicological characterisation of waste - results and experiences from a European ring test, Springer Ltd., New York, USA.

Mousavi SM, Bahmanyar MA, Pirdashti H (2013). Phytoavailability of some micronutrients (Zn and Cu), heavy metals (Pb, Cd), and yield of rice affected by sewage sludge perennial application. Communications in Soil Science and Plant Analysis 44.

Muñoz I, José Gómez M, Molina-Díaz A, Huijbregts MAJ, Fernández-Alba AR, García-Calvo E (2008). Ranking potential impacts of priority and emerging pollutants in urban wastewater through life cycle impact assessment. Chemosphere 74:37–44.

Munoz I, Jose Gomez-Ramos M, Aguera A, Francisco Garcia-Reyes J, Molina-Diaz A, Fernandez-Alba AR (2009). Chemical evaluation of contaminants in wastewater effluents and the environmental risk of reusing effluents in agriculture. Trac-Trends Anal Chem 28:676–694.

Muyen Z, Moore GA, Wrigley RJ (2011). Soil salinity and sodicity effects of wastewater irrigation in South East Australia. Agricultural Water Management 99:33–41.

Natal-da-Luz T, Ojeda G, Pratas J, Gestel C a. M Van, Sousa JP (2011). Toxicity to *Eisenia andrei* and *Folsomia candida* of a metal mixture applied to soil directly or via an organic matrix. Ecotox Environ Safe 74:1715–1720.

Natal-da-Luz T, Tidona S, Jesus B, Morais P, Sousa J (2009). The use of sewage sludge as soil amendment. The need for an ecotoxicological evaluation. Journal of Soils and Sediments 9:246–260.

Neuwoehner J, Escher BI (2011). The pH-dependent toxicity of basic pharmaceuticals in the green algae *Scenedesmus vacuolatus* can be explained with a toxicokinetic ion-trapping model. Aquatic Toxicology 101:266–275.

Newman MC (2009). Fundamentals of Ecotoxicology. Third edition, CRC Press, Taylor & Francis Group, New York, USA.

Oakes KD, Coors A, Escher BI, Fenner K, Garric J, Gust M, Knacker T, Küster A, Kussatz C, Metcalfe CD, Monteiro S, Moon TW, Mennigen JA, Parrott J, Péry AR, Ramil M, Roennefahrt I, Tarazona JV, Sánchez-Arguello P, Ternes TA, Trudeau VL, Boucard T, Der Kraak GJ Van, Servos MR (2010). Environmental risk assessment for the serotonin re-uptake inhibitor fluoxetine: Case study using the European risk assessment framework. Integr Environ Assess Manag 6:524–539.

Ochoa-Acuna HG, Bialkowski W, Yale G, Hahn L (2009). Toxicity of soybean rust fungicides to freshwater algae and Daphnia magna. Ecotoxicology 18:440–446.

OECD (Organisation for Economic Co-operaration and Development) (2000). ENV/JM/MONO(2000)6, OECD Series on Testing and Assessment Number 23, Guidance

Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures, OECD, Paris, France.

OECD (Organisation for Economic Co-operation and Development) (2006a). Test No. 208: Terrestrial plant test: Seedling emergence and seedling growth test. OECD Guidelines for the Testing of Chemicals. Paris, France.

OECD (Organisation for Economic Co-operation and Development) (2006b). Test No. 312: Leaching in Soil Columns. OECD Guidelines for the Testing of Chemicals. Paris, France.

Oehlmann J, Schulte-Oehlmann U, Tillmann M, Markert B (2000). Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part I: Bisphenol A and octylphenol as xeno-estrogens. Ecotoxicology 9:383–397.

Oh K, Yamada K, Asami T, Yoshizawa Y (2012). Synthesis of novel brassinosteroid biosynthesis inhibitors based on the ketoconazole scaffold. Bioorganic and Medicinal Chemistry Letters 22:1625–1628.

Oppel J, Broll G, Löffler D, Meller M, Römbke J, Ternes T (2004). Leaching behaviour of pharmaceuticals in soil-testing-systems: A part of an environmental risk assessment for groundwater protection. SciTotal Environ 328:265–273.

Otomo PV, Reinecke SA (2010). Increased cytotoxic and genotoxic tolerance of *Eisenia fetida* (Oligochaeta) to cadmium after long-term exposure. Ecotoxicology 19:362–368

Pandard P, Devillers J, Charissou AM, Poulsen V, Jourdain MJ, Férard JF, Grand C, Bispo A (2006). Selecting a battery of bioassays for ecotoxicological characterization of wastes. SciTotal Environ 363:114–125.

Paquin PR, Gorsuch JW, Apte S, Batley GE, Bowles KC, Campbell PGC, Delos CG, Toro DM Di, Dwyer RL, Galvez F, Gensemer RW, Goss GG, Hogstrand C, Janssen CR, McGeer JC, Naddy RB, Playle RC, Santore RC, Schneider U, Stubblefield WA, Wood CM, Wu KB (2002). The biotic ligand model: a historical overview. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 133:3–35.

Peng X, Huang Q, Zhang K, Yu Y, Wang Z, Wang C (2012). Distribution, behavior and fate of azole antifungals during mechanical, biological, and chemical treatments in sewage treatment plants in China. Sci.Total Environ. 426:311–317.

Perrodin Y, Christine B, Sylvie B, Alain D, Bertrand-Krajewski J-L, Cecile C-O, Audrey R, Elodie B (2013). A priori assessment of ecotoxicological risks linked to building a hospital. Chemosphere 90:1037–1046

Persoone G, Marsalek B, Blinova I, Torokne A, Zarina D, Manusadzianas L, Nalecz-Jawecki G, Tofan L, Stepanova N, Tothova L, Kolar B (2003). A practical and user-friendly toxicity

classification system with microbiotests for natural waters and wastewaters. EnvironToxicol 18:395–402

Petersen S., Henriksen K, Mortensen G., Krogh P., Brandt K., Sørensen J, Madsen T, Petersen J, Grøn C (2003). Recycling of sewage sludge and household compost to arable land: fate and effects of organic contaminants, and impact on soil fertility. Soil and Tillage Research 72:139–152

Peysson W, Vulliet E (2013). Determination of 136 pharmaceuticals and hormones in sewage sludge using quick, easy, cheap, effective, rugged and safe extraction followed by analysis with liquid chromatography-time-of-flight-mass spectrometry. J Chromatogr A 1290:46–61.

Postigo C, Barceló D (2015). Synthetic organic compounds and their transformation products in groundwater: Occurrence, fate and mitigation. Science of The Total Environment 503-504:32–47.

Power EA, Boumphrey RS (2004). International trends in bioassay use for effluent management. Ecotoxicology 13:377–398.

Prosser RS, Sibley PK (2015). Human health risk assessment of pharmaceuticals and personal care products in plant tissue due to biosolids and manure amendments, and wastewater irrigation. Environ Int 75:223–233.

Quednow K, Püttmann W (2007). Monitoring terbutryn pollution in small rivers of Hesse, Germany. Journal of Environmental Monitoring 9:1337.

Rademacher W (2000). Growth retardants: Effects on gibberellin biosynthesis and other metabolic pathways. Annual Review of Plant Physiology and Plant Molecular Biology 51:501–531.

Rajalekshmi KM, Jaleel CA, Azooz MM, Panneerselvam R (2009). Effect of triazole growth regulators on growth and pigment contents in *Plectranthus aromaticus* and *Plectranthus vettiveroids*. Advances in Biological Research 3:117–122.

Ramírez WA, Domene X, Ortiz O, Alcañiz JM (2008). Toxic effects of digested, composted and thermally-dried sewage sludge on three plants. Bioresource Technology 99:7168–7175.

Reemtsma T, Miehe U, Duennbier U, Jekel M (2010). Polar pollutants in municipal wastewater and the water cycle: Occurrence and removal of benzotriazoles. Water Research 44:596–604.

Reinecke AJ, Maboeta MS, Vermeulen LA, Reinecke SA (2002). Assessment of lead nitrate and mancozeb toxicity in earthworms using the avoidance response. Bulletin of Environmental Contamination and Toxicology 68:779–786.

Rendal C, Kusk KO, Trapp S (2011). Optimal choice of pH for toxicity and bioaccumulation studies of ionizing organic chemicals. EnvironToxicolChem 30:2395–2406

Rendal C, Trapp S, Kusk KO (2012). Critical evaluation and further development of methods for testing ecotoxicity at multiple pH using *Daphnia magna* and *Pseudokirchneriella subcapitata*. EnvironToxicolChem 31:1843–1852.

Richter E, Wick A, Ternes TA, Coors A (2013). Ecotoxicity of climbazole, a fungicide contained in anti-dandruff shampoo. Environmental Toxicology and Chemistry 32, 2816-2825.

Richter E, Roller E, Kunkel U, Ternes TA, Coors A (2015). Phytotoxicity of wastewater-born micropollutants – characterisation of three antimycotics and a cationic surfactant. Environmental Pollution. *submitted*

Römbke J, Egeler P (2009). Oligochaete worms for ecotoxicological assessment of soils and sediments (DH Shain, Ed.). Annelids in Modern Biology:225–238.

Rogers HR (1996). Sources, behaviour and fate of organic contaminants during sewage treatment and in sewage sludges. Science of The Total Environment 185:3–26.

Roig N, Sierra J, Nadal M, Martí E, Navalón-Madrigal P, Schuhmacher M, Domingo JL (2012). Relationship between pollutant content and ecotoxicity of sewage sludges from Spanish wastewater treatment plants. Science of The Total Environment 425:99–109

Rönnpagel K, Janßen E, Ahlf W (1998). Asking for the indicator function of bioassays evaluating soil contamination: Are bioassay results reasonable surrogates of effects on soil microflora? Chemosphere 36:1291–1304.

Rosenkrantz RT, Cedergreen N, Baun A, Kusk KO (2013). Influence of pH, light cycle, and temperature on ecotoxicity of four sulfonylurea herbicides towards Lemna gibba. Ecotoxicology 22:33–41.

Sabourin L, Rajab AJ Al, Chapman R, Lapen DR, Topp E (2011). Fate of the antifungal drug clotrimazole in agricultural soil. EnvironToxicolChem 30:582–587

Sarakinos HC, Bermingham N, White PA, Rasmussen JB (2000). Correspondence between whole effluent toxicity and the presence of priority substances in complex industrial effluents. Environmental Toxicology and Chemistry 19:63–71.

Sarkar B, Megharaj M, Xi Y, Krishnamurti GSR, Naidu R (2010). Sorption of quaternary ammonium compounds in soils: implications to the soil microbial activities. J Hazard Mater 184:448–456.

Scheffer/Schachtschabel: Lehrbuch der Bodenkunde (2010). Blume H-W, Brümmer GW, Horn R, Kandeler E, Kögel-Knabner I, Kretzschmar R, Stahr K, Wilke B-M, 16. Auflage, Spektrum Akademischer Verlag Heidelberg, Germany.

Scheurer M, Heß S, Lüddeke F, Sacher F, Güde H, Löffler H, Gallert C (2015). Removal of micropollutants, facultative pathogenic and antibiotic resistant bacteria in a full-scale

retention soil filter receiving combined sewer overflow. Environ Sci: Processes Impacts 17:186–196.

Schowanek, D., Carr R, David H, Douben H, Hall J, Kirchmann H, Patria L, Sequi P, Smith S, Webb S (2004). A risk-based methodology for deriving quality standards for organic contaminants in sewage sludge for use in agriculture - Conceptual Framework. Regulatory Toxicology and Pharmacology 40:227–251.

Seco JI, Fernández-Pereira C, Vale J (2003). A study of the leachate toxicity of metal-containing solid wastes using *Daphnia magna*. Ecotoxicology and Environmental Safety 56:339–350.

Seeland A, Oehlmann J, Müller R (2012). Aquatic ecotoxicity of the fungicide pyrimethanil: Effect profile under optimal and thermal stress conditions. Environmental Pollution 168:161–169.

Smolders E, Oorts K, Sprang P van, Schoeters I, Janssen CR, McGrath SP, McLaughlin MJ (2009). Toxicity of trace metals in soil as affected by soil type and aging after contamination: using calibrated bioavailability models to set ecological soil standards. Environ Toxicol Chem 28:1633–1642.

Spurgeon DJ, Lister L, Kille P, Pereira MG, Wright J, Svendsen C (2011). Toxicokinetic studies reveal variability in earthworm pollutant handling. Pedobiologia 54, Supplement:S217–S222.

Statistisches Bundesamt (2013). Umwelt Abwasserbehandlung – Klärschlamm – Ergebnisbericht 2010, Artikelnummer: 5322101109004, Wiesbaden, Germany.

Stravinskene ES, Grigor'ev YS (2012). The modifying effect of natural waters on the toxicity of heavy metals for algae. Water Resour 39:330–334.

Tang JYM, Busetti F, Charrois JWA, Escher BI (2014). Which chemicals drive biological effects in wastewater and recycled water? Water Research 60:289–299.

Tanoue R, Sato Y, Motoyama M, Nakagawa S, Shinohara R, Nomiyama K (2012). Plant uptake of pharmaceutical chemicals detected in recycled organic manure and reclaimed wastewater. J Agric Food Chem 60:10203–10211.

Teijon G, Candela L, Tamoh K, Molina-Diaz A, Fernandez-Alba AR (2010). Occurrence of emerging contaminants, priority substances (2008/105/CE) and heavy metals in treated wastewater and groundwater at Depurbaix facility (Barcelona, Spain). Sci Total Environ 408:3584–3595.

Ternes TA (1998). Occurrence of drugs in German sewage treatment plants and rivers. Water Research 32:3245–3260.

Ternes TA, Bonerz M, Herrmann N, Teiser B, Andersen HR (2007). Irrigation of treated wastewater in Braunschweig, Germany: An option to remove pharmaceuticals and musk fragrances. Chemosphere 66:894–904.

Ternes TA, Joss A, Siegrist H (2004). Scrutinizing pharmaceuticals and personal care products in wastewater treatment. Environmental Science and Technology 38:392A–399A

Thorpe KL, Cummings RI, Hutchinson TH, Scholze M, Brighty G, Sumpter JP, Tyler CR (2003). Relative potencies and combination effects of steroidal estrogens in fish. Environmental Science and Technology 37:1142–1149.

Topp E, Monteiro SC, Beck A, Coelho BB, Boxall ABA, Duenk PW, Kleywegt S, Lapen DR, Payne M, Sabourin L, Li H, Metcalfe CD (2008). Runoff of pharmaceuticals and personal care products following application of biosolids to an agricultural field. Sci Total Environ 396:52–59.

Trapp S (2000). Modelling uptake into roots and subsequent translocation of neutral and ionisable organic compounds. Pest Management Science 56:767–778.

Triebskorn R, Casper H, Scheil V, Schwaiger J (2007). Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). Analytical and Bioanalytical Chemistry 387:1405–1416.

Trösken ER, Scholz K, Lutz RW, Völkel W, Zarn JA, Lutz WK (2004). Comparative assessment of the inhibition of recombinant human CYP19 (aromatase) by azoles used in agriculture and as drugs for humans. Endocrine Research 30:387–394.

Tyagi VK, Lo S-L (2013). Sludge: A waste or renewable source for energy and resources recovery? Renewable and Sustainable Energy Reviews 25:708–728.

UBA (Bundesministerium für Umwelt, Naturschutz, Bau und Reaktorsicherheit) (2001). Grundsätze und Maßnahmen für eine vorsorgeorientierte Begrenzung von Schadstoffeinträgen in landbaulich genutzten Böden. Texte 59/01, ISSN 0722-186X, Berlin, Germany.

UBA (Bundesministerium für Umwelt, Naturschutz, Bau und Reaktorsicherheit) (2013). Water Resource Management in Germany. Part 1 – Fundamentals. Bonn, Germany.

von der Ohe PC, Dulio V, Slobodnik J, Deckere E De, Kühne R, Ebert R-U, Ginebreda A, Cooman W De, Schüürmann G, Brack W (2011). A new risk assessment approach for the prioritization of 500 classical and emerging organic microcontaminants as potential river basin specific pollutants under the European Water Framework Directive. Science of The Total Environment 409:2064–2077.

Valcarcel Y, Gonzalez Alonso S, Rodriguez-Gil JL, Gil A, Catala M (2011). Detection of pharmaceutically active compounds in the rivers and tap water of the Madrid Region (Spain) and potential ecotoxicological risk. Chemosphere 84:1336–1348

Verlicchi P, Zambello E (2014). How efficient are constructed wetlands in removing pharmaceuticals from untreated and treated urban wastewaters? A review. Science of The Total Environment 470–471:1281–1306.

Verlicchi P, Aukidy M Al, Galletti A, Petrovic M, Barceló D (2012). Hospital effluent: Investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment. SciTotal Environ 430:109–118.

Warren-Hicks WJ, Parkhurst BR, Moore DRJ, Teed RS, Baird RB, Berger R, Denton DL, Pletl JJ (2000). Assessment of whole effluent toxicity test variability: Partitioning sources of variability. Environmental Toxicology and Chemistry 19:94–104.

Waste Framework Directive, Directive 2008/98/EC of the European Parliament and of the Council of 19 November 2008 on waste and repealing certain directives. ABl. L 312 vom 22.11.2012, S. 3-30.

WFD (Water Framework Directive), Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy, OJ No. L 327, p. 1 ff.

WHG, Wasserhaushaltsgesetz vom 31. Juli 2009 (BGBl. I S. 2585), das zuletzt durch Artikel 2 des Gesetzes vom 15.November 2014 (BGBl. I S. 1724) geändert worden ist.

Wick A, Fink G, Ternes TA (2010). Comparison of electrospray ionization and atmospheric pressure chemical ionization for multi-residue analysis of biocides, UV-filters and benzothiazoles in aqueous matrices and activated sludge by liquid chromatography-tandem mass spectrometry. Journal of Chromatography A 1217:2088–2103.

Xu J, Wu LS, Chen WP, Chang AC (2010). Leaching potential of nonsteroidal anti-inflammatory drugs in soils. EnvironToxicolChem 29:800–807.

Zhang C, Tezel U, Li K, Liu D, Ren R, Du J, Pavlostathis SG (2011). Evaluation and modeling of benzalkonium chloride inhibition and biodegradation in activated sludge. Water Research 45:1238–1246.

Zarn J A, Brüschweiler BJ, Schlatter JR (2002). Azole fungicides affect mammalian steroidogenesis by inhibiting sterol 14a-demethylase and aromatase. Environ Health Perspect 111.

Zubrod JP, Bundschuh M, Feckler A, Englert D, Schulz R (2011). Ecotoxicological impact of the fungicide tebuconazole on an aquatic decomposer-detritivore system. Environmental Toxicology and Chemistry 30:2718–2724.

A. Annex

A.1. Publications as part of this thesis

A.1.1 Ecotoxicity of climbazole, a fungicide contained in anti-dandruff shampoo

Richter E, Wick A, Ternes TA, Coors A (2013): Environmental Toxicology and Chemistry 32, 2816-2825.

Author contributions:

Initials: Elisabeth Richter (ER), Arne Wick (AW), Thomas A. Ternes (TT), Anja Coors (AC).

Study design and planning:

ER: 70%; AC: 25%; AW: 5%.

Performance of the experiments and analyses:

ER: 90%, biological tests; AW: 10%, chemical analysis.

Data assembling and preparing of figures:

ER: 70%, biological and partially chemical results; AC: 20%, support in statistical evaluation; AW: 10%, assembling chemical results.

Data analysis and interpretation:

ER: 60%, analysis and interpretation of biological and chemical results; AC: 30%, AW, TT: 10%, discussion and further interpretation of results.

Introduction, results and discussion:

ER: 60%; AC: 20%; AW: 10%; TT: 10%.

SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 32, No. 12, pp. 2816–2825, 2013

Printed in the USA

ECOTOXICITY OF CLIMBAZOLE, A FUNGICIDE CONTAINED IN ANTIDANDRUFF SHAMPOO

ELISABETH RICHTER,*†‡ ARNE WICK,§ THOMAS A. TERNES,§ and ANJA COORS†
†ECT Oekotoxikologie, Flörsheim am Main, Germany
‡Goethe-University Frankfurt am Main, Department Aquatic Ecotoxicology, Frankfurt am Main, Germany
§Federal Institute of Hydrology, Koblenz, Germany

(Submitted 17 March 2013; Returned for Revision 24 May 2013; Accepted 19 August 2013)

Abstract: Emerging pollutants such as personal care products can reach the environment via effluents from wastewater treatment plants (WWTPs) and digested sludge. Only recently, the antidandruff agent and antimycotic climbazole was detected for the first time in a WWTP effluent with concentrations up to 0.5 μg/L. Climbazole acts as a C14-demethylase inhibitor (DMI) fungicide and thus has a high efficacy against fungi, but knowledge of its potential environmental impact is lacking. Therefore, the aim of the present study was to characterize climbazole's ecotoxicity by conducting standard biotests with organisms representing different trophic levels from the aquatic as well as the terrestrial ecosystems. It was found that the toxicity of climbazole is mostly similar to that of other DMI fungicides, whereas it proved to be particularly toxic to primary producers. The lowest median effective concentrations (EC50s) were determined for *Lemna minor*, at 0.013 mg/L (biomass yield), and *Avena sativa*, at 18.5 mg/kg soil dry weight (shoot biomass). Reduction of frond size in water lentils and shoot length in higher plants suggested an additional plant growth–retarding mode of action of climbazole. In addition, it was demonstrated here that for an ionizable compound such as climbazole, the soil pH can have a considerable influence on phytotoxicity. *Environ Toxicol Chem* 2013;32:2816–2825. © 2013 SETAC

Keywords: Cosmetics Ergosterol Biosynthesis Phytohormone Ionizing compounds

INTRODUCTION

Personal care products are consumed in private households world-wide and in often high quantities [1]. They include goods such as soaps, hair care formulations, skin care products, toothpastes, perfumes, and sunscreens [2]. In general, these products are designed to clean, protect, or change through external application the human body's appearance or odor [3]. Thus, unlike pharmaceuticals, most of them possess little biological activity and in general enter the sewage system unaltered [2,3]. Occasionally, they can also be released directly into recreational waters [2]. As municipal wastewater treatment plants (WWTPs) are primarily optimized to remove inorganic phosphorus and nitrogen species but not organic micropollutants, personal care products may eventually reach the environment via discharges of WWTP effluents and the use of digested sludge as fertilizer on agricultural land [4]. Although introduced to surface waters only at low concentration levels (ng/L range), personal care products still raise concern because aquatic organisms may be exposed to them on a chronic, multigenerational time scale and as complex mixtures [5]. Comprising a large variety of substances with often unknown environmental fates and effects or properties of concern, the ingredients of personal care products are considered emerging pollutants that need to be further investigated [2,6].

Recently, the antidandruff agent climbazole was monitored and detected for the first time in effluents of a conventional German WWTP at concentrations of approximately 0.5 μg/L; in raw wastewater and activated sludge, up to 1.4 μg/L and 1.2 μg/g total suspended solids, respectively, were found [7]. Climbazole is used in cosmetics as an antimycotic preservative and especially as an active ingredient of antidandruff hair care formulations up to a maximum content of 2.0%, equivalent to approximately 15 g/L in antidandruff shampoos [8]. Products containing climbazole are widely available in many countries, for example, in Western Europe and China [9]. Climbazole belongs to the group of imidazole fungicides and inhibits the biosynthesis of ergosterol, the major component of fungal plasma membranes, by noncompetitively blocking the active site of the enzyme lanosterol 14α-demethylase, also known as fungal CYP51 [10]. Depending on the dose, climbazole can also induce the production of cytochrome P450 and phase II–metabolizing enzymes in the liver of rats [10]. In efficacy studies it was shown that human toxicity is low for rinse-off application and that genotoxic, teratogenic, or mutagenic effects are unlikely [8,10]. However, ecological information on climbazole is limited to an aquatic toxicity data set provided by the supplier. As it affects the growth of the green alga *Pseudokirchneriella subcapitata* at very low concentrations (median effective concentration [EC50] of 0.087 mg/L, [8]), climbazole is classified as very toxic to aquatic organisms.

Azole antifungals have been used for the treatment of human mycoses since the 1960s and as agricultural fungicides since the 1970s [11]. Conazole fungicides, comprising triazoles and imidazoles, represent approximately one-third of agrochemical fungicides used [12]. Within the fungicide classification, azoles belong to the ergosterol biosynthesis inhibitors, forming the subgroup of sterol demethylase inhibitors, also called C14-demethylase inhibitor (DMI) fungicides. Climbazole, like other imidazoles, possesses a heterocyclic planar ring and occurs in 2 enantiomers. Because of its pK_a of 7.5 at neutral pH [7], both a neutral and a positively charged molecule are present, whereas at acidic pH the positively charged form prevails. In fact, the pK_a value of organic chemicals may strongly affect their toxicokinetics and sometimes toxicodynamics [13,14]. The algal toxicity of the beta-blocker propranolol, for example, can differ by a factor of 200 depending on pH [14].

All Supplemental Data may be found in the online version of this article.
* Address correspondence to e.richter@ect.de.
Published online 24 August 2013 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.2367

Because of the mode of action and presence in surface water of climbazole, it is highly relevant to study its ecotoxicological effects. As climbazole is found in WWTP effluents as well as in sewage sludge, it may reach both the aquatic and terrestrial ecosystems. Therefore, the aim of the present study was to characterize the ecotoxicological effects of climbazole on a range of aquatic as well as terrestrial organisms of different trophic levels. To this end, we determined species-specific effective concentrations for 5 aquatic and 5 terrestrial organisms in laboratory tests according to international standard guidelines. In addition, we examined the pH dependence of the aquatic toxicity and investigated the influence of soil pH and organic matter content on the terrestrial phytotoxicity. Finally, we compared the ecotoxicity and mode of action of climbazole with that of other DMI fungicides based on literature data.

MATERIALS AND METHODS

Test substance

Climbazole (see Table 1) was purchased from Tokyo Chemical Industry Co. The exact stereoisomeric composition of the batch used here was unknown.

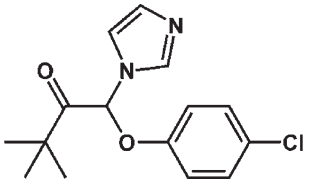
Biotest design and preparation of climbazole treatments

Selection of the test concentrations was based on preliminary range-finding tests (data not shown). Detailed test conditions are summarized in Supplemental Data, Table S1.

For aquatic biotests, stock solutions were prepared in the respective test media by ultrasound treatment for 15 min and stirring overnight in the dark at room temperature. Test solutions were prepared as dilutions of the stock solution directly before test start. Biotests were perfomed under static test conditions in glass vessels.

For terrestrial biotests, climbazole was added directly into the test substrate (soil) to obtain the appropriate test concentrations. To this end, the substance was pestled into a small amount of quartz sand and was then homogenized with the residual soil. In the case of *Arthrobacter globiformis*, however, the substrate was spiked with test solutions that were prepared as dilutions of an acetone stock solution. Two substrates were used: a natural sandy loam soil, LUFA 2.3 (LUFA Speyer), which is often used for plant tests; and an artificial soil with 5% peat according to Organization for Economic Cooperation and Development (OECD) guideline 207 [15,16]. For soil characteristics, see Supplemental Data, Table S3. For the terrestrial tests, all concentrations refer to soil dry weight.

Table 1. Physicochemical characteristics of climbazole



Molecular structure

CAS registry number	38083-17-9[a]
IUPAC nomenclature	1-(4-chlorophenoxy)-1-imidazol-1-yl-3,3 dimethyl-2-butanone[a]
Molecular weight	292.76 g/mol[a]
Melting point	96.8 °C[a]
Vapor pressure	2.1 E^{-007}(mm Hg, 25 °C)[a]
Water solubility	Up to 50 mg/L (25 °C, pH 7.8, empiric)
1-Octanol/water partition coefficient (log K_{OW})	3.33[b,c]
Dissociation constant (pK_a)	7.5 (weak base)[c]
Log K_{OW} corrected for ionization (log D_{OW})	Between 1.82 (pH 6.0) and 3.27 (pH 8.3)[d]

[a]Scientific Committee on Consumer Products [8].
[b]Referring to the neutral molecule.
[c]Wick et al. [7], from ALOGPS 2.1.
[d]Referring to lowest and highest test pH, calculated according to Schwarzenbach et al. [44].
CAS = Chemical Abstract Services; IUPAC = International Union of Pure and Applied Chemistry

Growth inhibition of water lentils after 7 d (OECD guideline 221)

According to OECD guideline 221 [17], *Lemna minor* L. was maintained as batch culture in modified Steinberg's medium at 24 °C ± 2 °C under continuous photosynthetically active radiation (PAR) of 85 μE/m^2 × s to 135 μE/m^2 × s. The same conditions applied to the biotest. Treatments were run with 3 replicates (control with 6) in crystallizing dishes containing 200 mL test volume and 4 *Lemna* colonies (12 fronds). After 7 d, frond number and frond dry weight were recorded, and biomass yield and growth rate of frond number were evaluated as endpoints.

Growth inhibition of diatoms and green algae after 72 h (OECD guideline 201)

According to OECD guideline 201 [18], *Navicula pelliculosa* (diatom) and *Pseudokirchneriella subcapitata* (green alga) were maintained as batch cultures in AAP medium and Kuhl and Lorenzen medium, respectively, at 21 °C to 24 °C under continuous PAR of 60 μE/m^2 × s to 120 μE/m^2 × s. The same conditions applied to the biotest. Treatments were run with 3 replicates (control with 6) in Erlenmeyer flasks containing 100 mL test volume. For diatoms, test solutions were inoculated with relatively high cell densities (4×10^4 cells/mL) to obtain sufficient growth over the test period; this density caused a pH increase in the control of more than the recommended 1.5 units. However, this increase is not considered to invalidate the test. For green algae, test solutions were inoculated with cell densities of 0.5×10^4 cells/mL. Cell density as a surrogate parameter for biomass was estimated via autofluorescence measurements of 200-μL aliquots using a spectral fluorometer (Tecan multiplate reader, Tecan Group). As endpoints, growth rate and biomass yield were evaluated.

Mortality of fish embryos after 48 h (OECD draft guideline 2006)

According to OECD draft guideline 2006 [19], *Danio rerio* embryos from in-house cultures were exposed to test solutions within 1 h after fertilization. The test was performed in reconstituted water [20], supplemented with 0.1% artificial seawater. Treatments and control were run with 4 replicates (each including 10 eggs) in crystallizing dishes containing 100 mL of test solution at 26 °C ± 1 °C and low light conditions (~300 lx). Oxygen saturation was 100% throughout the test. Lethal effects (coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail bud from the yolk sac, and lack of heartbeat) were evaluated after 48 h.

Mortality of water fleas after 48 h (OECD guideline 202)

According to OECD guideline 202 [21], *Daphnia magna* were kept in Elendt M4 medium and subjected to a 16:8-h light: dark cycle at low light conditions (~450 lx); temperature was maintained at 22 °C ± 1 °C. The same conditions applied to the biotest. Treatments and control were run with 4 replicates (each including 5 daphnids) in glass beakers containing 150 mL of test

solution. No feed was supplied, and oxygen saturation was 100% throughout the test. Immobility after 48 h was evaluated as a surrogate parameter for mortality.

Growth inhibition of terrestrial plants after 17 d (ISO 11269-2)

According to International Organization for Standardization (ISO) guideline 11269-2 [22], a standard dose–response test with certified seeds of turnip (*Brassica napus*) and oats (*Avena sativa*) was conducted in 2 soils. As substrates, LUFA 2.3 and artificial soil (5% peat) were used. Treatments and controls (1 for each soil) were run with 4 replicate pots, containing 600 g ± 5 g (*B. napus*) and 300 g ± 5 g soil dry weight (*A. sativa*). Per pot, 10 seeds were sown and after determining the germination rate, seedlings were reduced to 5 representive plants. Plants were cultivated in a controlled environment chamber with 16:8-h light:dark cycle (∼230 $\mu E/m^2 \times s^1$ PAR) for 17 d; temperature was maintained at 22 °C ± 10 °C; watering was provided as necessary, and a liquid fertilizer was supplemented once. As endpoints, germination rate (emergence), shoot length, and shoot fresh weight (biomass) were evaluated.

Inhibition of reproduction of enchytraeids after 4 wk (OECD guideline 220)

According to OECD guideline 220 [23], *Enchytraeus bigeminus* was cultured in-house in agar at 20 °C ± 5 °C and fed oatmeal weekly ad libitum. Synchronized adults were used for the test without acclimatization. As substrate, artificial soil was used, and humidity was set to 55% of maximum water-holding capacity (WHK_{max}) and readjusted weekly. Treatments and control were run with 4 replicate glass vessels containing 20 g ± 5 g dry weight and 10 adult worms. Oatmeal was supplemented as food with 50 mg per replicate at test start and later with 25 mg/wk. The test was conducted in a controlled environment chamber under a 16:8-h light:dark cycle of 400 lx to 800 lx at 20 °C ± 2 °C. After 28 d, adult and juvenile worms were counted using a binocular microscope after Bengal red staining overnight; as endpoint, reproduction (number of juveniles) was evaluated.

Inhibition of reproduction of springtails after 4 wk (OECD guideline 232)

According to OECD guideline 232 [24], the collembolan *Folsomia candida* was cultured in-house on plaster of Paris (calcium sulfate with activated charcoal) in the dark at 20 °C ± 4 °C; synchronized juveniles 9 d to 10 d old were used for the test. As substrate, artificial soil was used, and humidity was set to 50% WHK_{max} and readjusted weekly. Treatments and control were run with 4-replicate and 8-replicate glass vessels, respectively, containing 30 g ± 5 g dry weight and 10 collembolans. Yeast was supplemented at approximately 5 mg per glass vessel at test start and after 14 d. The test was conducted in a controlled environment chamber in the dark at 20 °C ± 4 °C. After 28 d, adult and juvenile collembola were counted using a binocular microscope after ink staining; as endpoint, reproduction was evaluated.

Inhibition of dehydrogenase activity in soil bacteria (draft ISO guideline 10871)

For the test according to draft ISO guideline 10871 [25] with the soil bacterium *Arthrobacter globiformis*, quartz sand was used as substrate and spiked with test solutions, which were prepared as dilutions of an acetone stock solution. In addition to the water control, an acetone control and a positive control at 600 mg/kg benzyl dimethyl hexadecylammonium chloride (CAS RN 122-18-9; Sigma-Aldrich) were tested; the latter was expected to result in a 40% to 60% effect. After evaporation of acetone, treatments were adjusted to 33% moisture with deionized water and weighed into translucent microtiter plates (24-well plate, Greiner Bio-One) at 0.6 g fresh weight per well and 4 replicates per treatments and controls. Plates were pasteurized twice for 15 min, bacteria inoculum was added at 0.4 mL per well, and plates were incubated at 30 °C ± 2 °C for 2 h on a horizontal shaker in the dark. Resazurin solution (CAS RN 62758-13-8; 4.5 mg/L; Sigma-Aldrich) was added at 0.8 mL per well, and fluorescence of the generated resofurin was measured for 60 min at 15-min intervals using a spectral fluorometer (excitation at 535, adsorbance at 590 nm). As endpoint, inhibition of dehydrogenase activity compared with the acetone control was determined based on the fluorescence slopes between 15 min and 45 min.

Growth inhibition of plants (B. napus) in soils with varying peat content and pH (ISO guideline 11269-2)

A 2-factorial experiment with *B. napus* was conducted based on the outcome of the above-mentioned dose–response tests to examine the influence of peat content and soil pH on the toxicity of climbazole. International guidelines for plant growth inhibition tests [22,26] stipulate a maximum organic matter content of 3.0%, whereas for pH a range of 5.0 to 7.5 is accepted. Three artificial soils were prepared for this experiment, each with 20% kaolin clay and either 5.0% peat with 75.0% quartz sand, 7.5% peat with 72.5% quartz sand, or 10.0% peat with 70.0% quartz sand. The organic carbon content of these soils was determined to be 2.0% ± 0.5%, 3.3% ± 0.4%, and 3.6% ± 0.2%, respectively. Each soil batch was devided into 3 parts, and the pH was adjusted (calcium carbonate, sodium hydroxide [5 mol/L] and/or sulfuric acid [0.5 mol/L]) to 5.0 ± 0.1, 7.5 ± 0.1, and 7.7 ± 0.1 (pH values close to climbazole's pK_a of 7.5), resulting in a total of 9 soils. Then each soil was divided into 2 portions, 1 as control and 1 spiked with 100 mg climbazole/kg soil dry weight. The test was run with 4 replicate pots (425 g ± 5 g soil dry wt). Per pot, 10 seeds were sown and after determining the emergence rate, seedlings were reduced to 5 representative plants. Test conditions and endpoints were the same as for the dose–response test; plants were harvested after 14 d.

Chemical analysis of aquatic test solutions

Samples for chemical analysis were taken at the start of the tests and at the end, either from all test concentrations (*N. pelliculosa*, *P. subcapitata*) or from control, lowest, and highest test concentration (other biotests). Samples were stored frozen prior to analysis. Recovery of climbazole (%) was calculated based on the measured amount in relation to the nominal concentration. The analytical methods used for the analysis of climbazole were mainly based on those described in detail in Wick et al. [7]. In brief, aqueous samples were spiked with climbazole-d4 (chemical purity: 96%, isotopic purity: 97%; Toronto Research Chemicals) as surrogate standard and filtered through 0.45-μm syringe filters made of regenerated cellulose (Spartan, Whatman). Samples from nominal climbazole concentrations below 10 μg/L were extracted by solid-phase extraction (SPE) using OASIS HLB catridges (200 mg; Waters). Samples from nominal concentration above 10 μg/L were directly measured without previous enrichment by SPE. Detection was accomplished by a liquid chromatography–electrospray ionization tandem mass spectrometry system consisting of a binary liquid chromatography pump (Agilent 1200) and a tandem mass spectrometer (API 4000, AB Sciex). The mass spectrometer was

operated in the positive ionization mode. Quantification was done using internal standard calibration, and the limit of quantification was 1 ng/mL.

Statistical analysis

Results of the standard biotests were modeled as concentration–response curves in the free software R version 2.11.1 using the drc package [27,28]. Effects (as percentage of control for continuous data and as proportion for binomial data) were fitted against log-transformed concentrations (x) using the 4-parameter log–logistic model (function LL.4) given in Equation 1, with the parameter *c* relating to the lower and *d* relating to the upper limit of the curve and *b* relating to the slope. The EC50 was directly iteratively determined as the model parameter

$$f(x) = c + \frac{d - c}{1 + \exp(b(\log(x) - \log(\mathrm{EC50})))} \quad (1)$$

Whereas for the continuous response data obtained for *L. minor*, the 4-parameter model was used, for *N. pelliculosa*, *P. subcapitata, B. napus*, and *A. sativa*, 3 parameters were estimated with the lower limit fixed at 0 (function LL.3). For the binomial response data (*D. rerio* and *D. magna* biotests), the lower and upper limits were fixed at 0 and 1, respectively (function LL.2). Further effect concentrations, EC20 and EC10, were estimated by the ED function in drc and confidence intervals (95%) for all point estimates were determined using the delta method in drc.

Concentration–response modeling was based on nominal test concentrations. In addition, the nominal EC50 values were corrected by the recovery (mean of measured concentrations in initial and aged test solutions).

For the climbazole plant growth inhibition test in 9 different soils, results were expressed as growth inhibition in the treated soil compared with the respective untreated soil for the endpoints emergence, shoot length, and shoot biomass. These data (% inhibition) were then evaluated in STATISTICA (Ver 10) for their dependence on the fixed effects soil peat content and soil pH and the interaction of both factors. To this end, two-way analyses of variance (ANOVAs) were performed for each endpoint. Assumptions of normal error distribution and variance homogeneity were met as verified by visual inspection and Levene's test ($\alpha = 0.01$). Only for biomass was variance homogeneity poor due to an exceptionally small variance in the 6 soils with high pH. The effect size eta-squared (η^2), which can be interpreted as percentage of variance accounted for by a factor or model [29], was calculated by dividing the sum of squares (*SS*) of the factor (peat, pH, or peat × pH) by the total sums of squares of the two-way ANOVA

$$\eta^2 = \frac{SS_{between}}{SS_{total}} \quad (2)$$

Estimation of pH-dependent partitioning of climbazole

For each individual aquatic biotest and the plant dose–response tests, we calculated the fraction of the neutral molecule [f(B)] applying the Henderson–Hasselbach equation (Equation 3) for the mean pH over initial and aged test concentrations and using climbazole's pK_a (7.5)

$$f(B) = \frac{1}{1 + 10^{(pK_a - pH)}} \quad (3)$$

RESULTS

Validity of standard tests

The validity criteria of the respective test guidelines were met in all biotests conducted here (Supplemental Data, Table S2).

Effects of climbazole on aquatic organisms

All tested aquatic species were negatively affected by climbazole in a concentration-dependent manner covering at least 10% to 50% effect (Table 2). The highest toxicity was observed in *L. minor*, and decreased in the order of diatoms, green algae, zebrafish embryos, and *D. magna*.

Climbazole toxicity toward *D. magna* and *D. rerio* embryos was moderate, with an EC50 of 15.9 mg/L for immobility and an EC50 of 8.20 mg/L for mortality. Effects on green algae and

Table 2. Toxicity of climbazole toward aquatic organisms[a]

					Model parameters (SE)[b]		
Test organism	Endpoint	EC10 (CI) (mg/L)	EC20 (CI) (mg/L)	EC50 (CI) (mg/L)	*b*	*c*	*d*
Lemna minor	Biomass yield	0.0056 (0.0010 – 0.0102)	0.0088 (0.0040 – 0.0136)	0.0190 (0.0123 – 0.0256)	1.805 (0.603)	–8.081 (1.423)	12.079 (0.674)
	Growth rate frond number	0.0112 (0.0078 – 0.0146)	0.0169 (0.0131 – 0.0206)	0.0339 (0.0298 – 0.0380)	1.984 (0.219)	Fixed at 0	0.398 (0.005)
Navicula pelliculosa	Biomass yield	0.0641 (0.0413 – 0.0869)	0.0885 (0.0666 – 0.1104)	0.1536 (0.1305 – 0.1766)	2.515 (0.467)	Fixed at 0	214.512 (5.057)
	Growth rate	0.1083 (0.0875 – 0.1291)	0.1559 (0.1416 – 0.1702)	0.2906 (0.2293 – 0.3519)	2.226 (0.393)	Fixed at 0	1.337 (0.011)
Pseudokirchneriella subcapitata	Biomass yield	0.0287 (0.0069 – 0.0505)	0.0603 (0.0261 – 0.0944)	0.2144 (0.1519 – 0.2769)	1.093 (0.147)	Fixed at 0	141.921 (4.710)
	Growth rate	0.3147 (0.2226 – 0.4069)	0.5144 (0.4070 – 0.6217)	1.191 (1.069 – 1.314)	1.650 (0.147)	Fixed at 0	0.078 (0.001)
Danio rerio	Mortality	1.07 (–0.94 – 3.08)	2.272 (–0.8843 – 5.429)	8.20 (–0.8721 – 17.27)	–1.080 (0.481)	Fixed at 0	Fixed at 1
Daphnia magna	Immobility	9.66 (3.27 – 16.10)	11.634 (5.479 – 17.79)	15.99 (9.152 – 11.83)	–4.358 (2.305)	Fixed at 0	Fixed at 1

[a]Results from standard laboratory biotests. Test organisms, most sensitive endpoints, and 10%, 20%, and 50% effective concentrations (EC10, EC20, and EC50, respectively) based on nominal test concentrations with 95% confidence intervals (CI).
[b]Concentration–response curves fitted by log–logistic model with parameter *b* relating to the slope of the curve, parameter *c* being the lower, and parameter *d* the upper limit.
SE = standard error.

diatoms were observed approximately 1 order of magnitude lower, with EC50 values for yield of 0.214 mg/L and 0.154 mg/L, respectively. The water lentil *L. minor* exhibited the lowest EC50, at 0.019 mg/L for biomass yield. Observed symptoms in *L. minor* were stunted colony growth and darker green frond color, whereas in both algae, the characteristic crescent-shaped or oval cell appearance was lost.

Chemical analysis of the test solutions confirmed that recovery of nominal climbazole concentrations was moderate for all aquatic biotests, at 68% to 85% for initial and 62% to 74% for aged test solutions (Table 3). However, based on initially measured test concentrations, recovery at test end reached 81% to 99%. Hence, there was only a little loss of climbazole during the static exposure. Based on mean recovery (averaged over test duration), EC50 values were corrected for each aquatic biotest, resulting in corresponding lower effective concentrations. The lowest recovery-corrected EC50 of 0.013 mg/L was observed for biomass yield of *L. minor*.

Partitioning of climbazole in aquatic biotests

With respect to the aquatic biotests, no obvious correlation between the neutral molecule fraction of climbazole and its toxicity could be found. Figure 1 shows the nominal EC50 of each organism and the pH-dependent fraction of the neutral molecule in the respective test medium. The (calculated) pK_a of climbazole is 7.5, that is, at pH 7.5 half of the molecules are protonated (cationic form) and half are deprotonated (neutral form). The relatively high EC50s for *D. rerio* and *D. magna* were determined in test media with neutral fractions of 50% and 61%, respectively. Approximately similar EC50 values were determined, however, for the 2 algae species *N. pelliculosa* and *P. subcapitata*, although the neutral molecule fractions were different at 86% and 3%, respectively. In the test medium of *L. minor*, the species with the lowest EC50, only 5% of climbazole was present in the neutral form.

Effects of climbazole on terrestrial organisms

In the terrestrial tests, climbazole did not affect reproduction in the collembola *F. candida* and the enchytraeid *E. bigeminus* up to concentrations of 1000 mg/kg soil dry weight (Table 4). Climbazole exhibited low toxicity toward the soil bacterium *A. globiformis*, with an EC50 of 456 mg/kg soil dry weight for inhibition of dehydrogenase activity. In contrast, growth of the plants *A. sativa* (oats) and *B. napus* (turnip) proved to be very sensitive to climbazole, reflected in clear concentration–response curves for the endpoints shoot length and shoot biomass. Emergence was not significantly affected at concentrations up to 100 mg/kg soil dry weight and 316 mg/kg soil dry weight in LUFA 2.3 and artificial soil, respectively. Observed symptoms in plants were retarded, stunted growth of the leaves

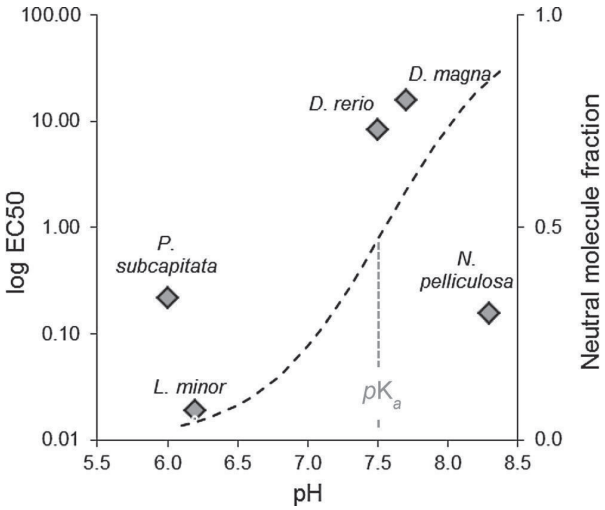


Figure 1. Overview of climbazole's nominal median effective concentration (EC50) determined in aquatic biotests, the pH of the respective test medium, and the estimated neutral molecule fraction at the given pH, calculated by the Henderson–Hasselbach equation. The diamonds mark the EC50 referring to the logarithmic left ordinate and the pH of each test medium on the abscissa. The dashed curve referring to the right ordinate depicts the estimated speciation of climbazole at the test medium's pH. At pH 7.5, climbazole has its pK_a where the cationic equals the neutral molecule fraction.

and especially the shoot as well as a darker green color than normal. Effective concentrations for shoot length (not shown here) were always slightly higher than those for biomass. The lowest EC50 for biomass yield was determined with 18.5 mg/kg soil dry weight for *A. sativa* grown in LUFA 2.3 soil. The same EC50 for *B. napus* was slightly higher, at 30.7 mg/kg soil dry weight. In contrast, when plants were grown in artificial soil, phytotoxicity was less pronounced, resulting in EC50s for *B. napus* of 45.5 mg/kg soil dry weight and for *A. sativa* of 83.8 mg/kg soil dry weight. According to Equation 3, the climbazole partitioning in the 2 soils should yield 2% neutral molecule fraction in artificial soil and 14% in LUFA soil.

Phytotoxicity to B. napus in soils with varying pH and peat content

In all 9 soils, plants developed normally in the respective untreated control. The influence of soil properties on the phytotoxicity of 100 mg/kg soil dry weight climbazole to *B. napus* is depicted in Figure 2. Results regarding the endpoints seedling emergence, shoot length, and biomass are shown as inhibition in the climbazole-treated soil compared with the respective untreated soil. Climbazole effects differed greatly in the soils, especially when comparing the more acidic with the more basic soils, where plant growth was almost completely

Table 3. Recovery of climbazole in aquatic biotests and recovery-corrected median effective concentration (EC50)[a]

Test organism and endpoint	Recovery of nominal concentrations (%)			Recovery of initial concentrations (%)	Nominal EC50 (mg/L)	EC50 corrected for mean recovery (mg/L)
	Initial	Aged	Mean			
Lemna minor	79 ± 23	62 ± 13	70 ± 18	80 ± 8	0.0190	0.0134
Navicula pelliculosa	78 ± 1	74 ± 3	76 ± 2	94 ± 4	0.154	0.116
Pseudokirchneriella subcapitata	85 ± 15	68 ± 4	76 ± 9	81 ± 8	0.214	0.163
Danio rerio	71 ± 3	69 ± 5	70 ± 4	97 ± 4	8.20	5.75
Daphnia magna	68 ± 13	68 ± 15	68 ± 14	99 ± 4	16.0	10.9

[a]Recovery of nominal concentrations in test solutions at test start (initial) and end (aged) and recovery of measured initial concentrations at test end, shown as means with standard deviations. All 5 test solutions were analyzed for *N. pelliculosa* and *P. subcapitata*, only the lowest and highest test solutions were analyzed for *L. minor*, *D. rerio* and *D. magna*.

Table 4. Toxicity of climbazole toward terrestrial organisms[a]

Test organism	Endpoint	EC10 (CI) (mg/kg soil dry wt)	EC20 (CI) (mg/kg soil dry wt)	EC50 (CI) (mg/kg soil dry wt)	Model parameters (SE)[b]		
					b	*c*	*d*
Avena sativa in LUFA	Biomass yield	4.9 (2.7 – 7.2)	8.0 (5.4 – 10.7)	18.5 (13.5 – 23.5)	1.66 (0.28)	Fixed at 0	0.93 (0.023)
Brassica napus in LUFA	Biomass yield	15.0 (0.9 – 29.0)	19.4 (2.0 – 37.0)	30.7 (1.5 – 59.9)	3.06 (1.20)	Fixed at 0	2.29 (0.055)
Avena sativa in artifical soil[c]	Biomass yield	22.1 (15.1 – 29.2)	36.2 (27.7 – 44.6)	83.8 (72.3 – 95.4)	1.65 (0.16)	Fixed at 0	1.06 (0.02)
Brassica napus in art. soil	Biomass yield	10.5 (5.8 – 15.1)	18.0 (12.1 – 24.0)	45.5 (37.5 – 53.6)	1.50 (0.17)	Fixed at 0	2.59 (0.06)
Arthrobacter globiformis	Dehydrogenase activity	400 (–361 – 1161)	420 (–207 – 1047)	456 (92 – 820)	16.99 (71.86)	23.25 (50.14)	526.95 (17.20)
Enchytraeus bigeminus	Reproduction	n.d.	n.d.	>1000	n.d.	n.d.	n.d.
Folsomia candida	Reproduction	n.d.	n.d.	>1000	n.d.	n.d.	n.d.

[a]Results from standard laboratory biotests. Test organisms, most sensitive endpoints, and 10%, 20%, and 50% effective concentrations (EC10, EC20, and EC50, respectively) based on nominal test concentrations with 95% confidence intervals (CI).
[b]Concentration–response curves fitted by log–logistic model, with parameter *b* relating to the slope of the curve, parameter *c* being the lower, and parameter *d* the upper limit.
[c]Artificial soil according to Organization for Economic Co-operation and Development test no. 207 with 5% peat.
SE = standard error; LUFA = standard soil LUFA 2.3; n.d. = not determined.

inhibited. According to Equation 3, the fraction of the neutral climbazole molecule increased from 1% at pH 5.5, to 50% at pH 7.5, to 61% at pH 7.7. Results of the 2-factorial ANOVAs (Table 5) confirmed that the influence of the soil pH was highly significant ($p < 0.001$) for all 3 endpoints, whereas the influence of the soil peat content was significant only for emergence ($p < 0.001$). Also, the effect size indicated a strong contribution of the factor pH to phytotoxicity, with eta-squared >0.7 for all endpoints, whereas the effect size of peat content proved to be only marginal, especially for shoot length and biomass (eta-squared values of 0.003 and 0.005, respectively). An interaction of the factors pH and peat was found only for emergence, whereas the strong influence of pH on the growth-inhibiting effects of climbazole was independent of the peat content of the soil. The observed effects in this test were very similar to those of the concentration–response test with *B. napus* regarding the treatment at 100 mg/kg soil dry weight climbazole in LUFA 2.3 and artificial soils, which underlines the reliability of the overall results.

DISCUSSION

Climbazole toxicity and mode of action in nontarget organisms

The DMI fungicides such as climbazole can be applied as systemic fungicides in agriculture or orally administered antimycotic drugs in medicine because their binding to the fungal lanosterol 14α-demethylase (CYP51) is considered to be relatively selective [12]. However, in the present study it was demonstrated that climbazole negatively affects a range of other organism groups, in particular aquatic and terrestrial primary producers. Among the aquatic species tested, climbazole proved to be very toxic to algae and water lentils, with the lowest EC50 being 0.019 mg/L and the EC10 being 0.005 mg/L in *L. minor*. For the terrestrial habitat, climbazole toxicity was highest toward plants with an EC50 of 18.5 mg/kg soil dry weight and an EC10 of 4.9 mg/kg soil dry weight in *A. sativa*. Based on the analytically measured test concentrations, effective concentrations are even lower, that is, for *L. minor* an EC50 of 0.0134 mg/L.

Nontarget organism effects of DMI fungicides originate from the fact that the amino acid sequence of CYP51 is not unique to fungi, but is highly conserved throughout all eukaryotic phyla, where it is likewise involved in biosynthesis of sterols [30,31]. Bulk sterols not only regulate membrane fluidity and permeability, but also serve as precursors for molecules controlling developmental processes such as mammalian steroid hormones, plant brassinosteroid hormones, and insect ecdysteroids [32]. Therefore, DMI substances may not only harm fungi but may also disturb membranes in unicellular organisms (like bacteria and algae), lower cholesterol production in animals (like *Daphnia* and fish), and interact with plant growth [32].

Growth retardation effects of climbazole in higher plants

In our experiments, effects on water lentils, which are aquatic macrophytes belonging to the monocotyledonous Araceae, were visible as dwarfed fronds of darker green than normal and inhibited colony formation. The effective concentrations in *L. minor* were lower than in algae by 1 order of magnitude. Likewise, the monocotyledonous oats (*A. sativa*) and the dicotyledonous rape (*B. napus)* exhibited stunted stem and leaf growth in the terrestrial plant tests.

That agricultural DMI fungicides affect agricultural plants has been observed since they were first marketed. Positive effects are reported, such as increased root growth and increased cold and drought tolerance, but also negative effects, such as reduced germination rates, shortened stem internodes, smaller and darker green leaves, and inhibited seed production [30]. For some fungicides, their additional growth-regulating action is intentionally applied in crop management [30]. In fact, synthetic plant growth retardants, which aim at reducing plant height in agriculture and horticulture by inhibiting gibberellin biosynthesis, comprise a range of N-heterocyclic compounds such as pyrimidines, triazoles, or imidazoles [33].

On the physiological level, the plant growth retardation property of DMI fungicides has been attributed to 2 modes of action; in addition to affecting sterol biosynthesis, they can inhibit the *ent*-kaurene hydroxylase and thereby block the biosynthesis of the phytohormone gibberellin [33]. Both sterols and gibberellins are derivates of the terpene metabolism and are vital for normal plant growth, but the phytohormone balance is more sensitive to disturbances than the sterol composition of the membrane [34]. Apparently, several DMI fungicides such as triadimefon or paclobutrazol exhibit both modes of action. Which of the 2 processes dominates seems to depend on the structural similarity to lanosterol or *ent*-kaurene, the respective intermediates of sterol and gibberellin biosynthesis [33]. In the macrophyte *Lemna*, gibberellins are also known to regulate growth processes [35]. In conclusion, we deduce from the observed symptoms in higher plants that climbazole interacts with sterol biosynthesis, but in particular that it very likely inhibits biosynthesis of the phytohormone gibberellin. However, for a final confirmation further studies are needed.

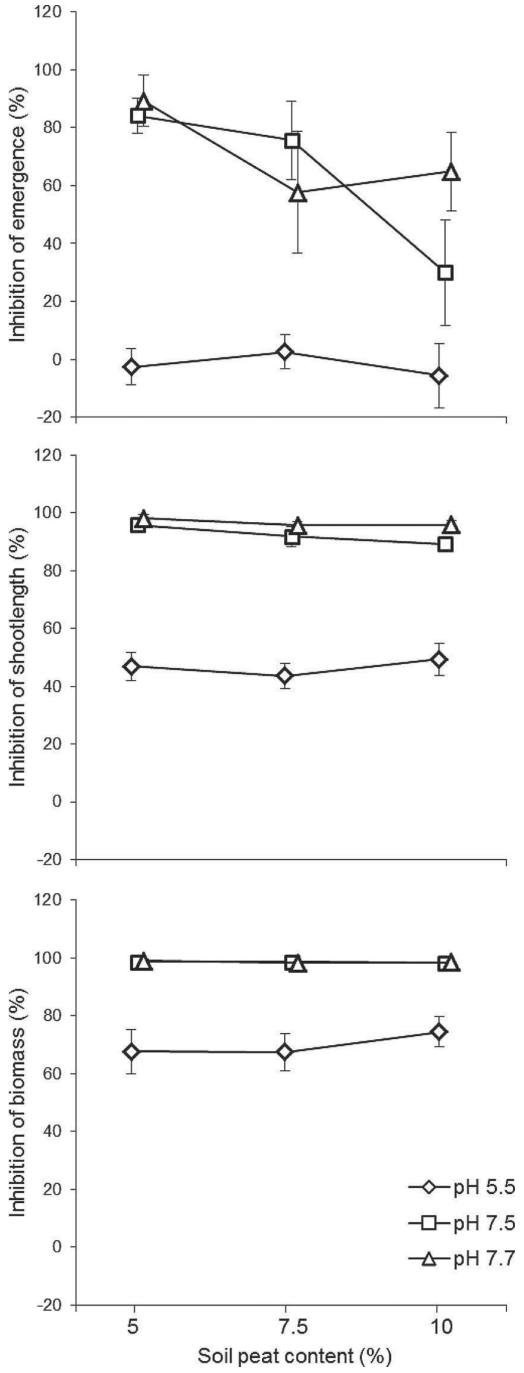


Figure 2. Growth inhibition of *Brassica napus* at 100 mg climbazole/kg soil dry weight in 9 artificial soils at varying soil pH (5.5, 7.5, and 7.7) and peat content (5%, 7.5%, and 10%). Seedling emergence (top), shoot length (middle), and shoot biomass (bottom). The results, expressed as percentage of inhibition compared with respective untreated control, are means with standard deviation ($n = 4$ replicates).

Table 5. Impact of the factors soil peat content and soil pH on the toxicity of climbazole toward *Brassica napus*[a]

Test endpoint	Soil factor	Degrees of freedom	Sum of squares	*F* value	*p* value	Effect size (η^2)[b]
Emergence						
	Peat	2	4446	13.7	<0.001	0.086
	PH	2	38 202	118	<0.001	0.739
	Peat × pH	4	4679	7.23	<0.001	0.099
	Error	27	4370			
Shoot length						
	Peat	2	64.7	3.04	0.064	0.003
	PH	2	18 409	865	<0.001	0.976
	Peat × pH	4	104	2.43	0.072	0.005
	Error	27	287			
Shoot biomass						
	Peat	2	36.8	1.29	0.292	0.005
	PH	2	6545	229	<0.001	0.927
	Peat × pH	4	92.2	1.61	0.200	0.013
	Error	27	386			

[a]Effects of 100 mg climbazole/kg soil dry weight on *Brassica napus* grown in 9 artificial soils varying in soil pH and peat content. Results of the two-way analysis of variances, $n = 4$.
[b]Effect size values calculated according to Equation 2.

Aquatic toxicity of climbazole in comparison with that of other DMI fungicides

From our results, the aquatic ecotoxicity of climbazole can be classified as very toxic to *Lemna* and algae, toxic to fish, and harmful to *Daphnia*. To relate climbazole toxicity to that of other DMI fungicides, we performed a literature review based on substance assessment reports by the European Comission [36] and the US Environmental Protection Agency [37]. Most of the available effect studies were conducted with the standard organisms green algae, daphnids, and fish. Although study details are patchy, the EC50s for comparable endpoints of 5 DMI fungicides, 1 plant growth retardant, and climbazole are compiled in Figure 3. This reveals that acute aquatic toxicity of climbazole lies in the same range as that of other DMI fungicides. However, with respect to *Lemna*, climbazole is more toxic than the other substances assessed by more than 1 order of magnitude. For green algae, only 2 substances exhibit higher toxicity than climbazole, and 1 is also more toxic to diatoms. In algae, the effects of DMI fungicides are likely to be caused by an alteration in sterol composition, which disturbs membrane fluidity and inhibits cell division [34]. Data on primary producers appear to diverge more than those for heterotrophic organisms (Figure 3). These variances could originate from species-specific differences in sensitivity because among algae and *Lemna*, various species were included in the data set, whereas biotests with *Daphnia* and fish were mostly conducted with the same species. Besides, tests on primary producers also assessed sublethal endpoints, whereas those on *Daphnia* and fish employed acute endpoints of mortality. Furthermore, the tests were probably conducted at different pH ranges, although this is documented incompletely. In any case, because the fungicides tested are very weak bases, they should be uncharged under average test conditions. Another explanation may be that the specific mode of action of each DMI fungicide differs in primary producers, that is, some of them do not inhibit gibberellin biosynthesis. Overall, our findings are in concordance with the review by Maltby et al. [31], who summarize as follows: the toxicity of 6 DMI fungicides toward 59 taxa is highest in primary producers followed by invertebrates and fish.

Influence of protonation of climbazole on its aquatic toxicity

Differences in the toxicity of a substance to several organism groups may result from its species-specific mode of action. However, in the case of organic substances that change their speciation at a realistic pH (2–9), the test media's pH is a critical

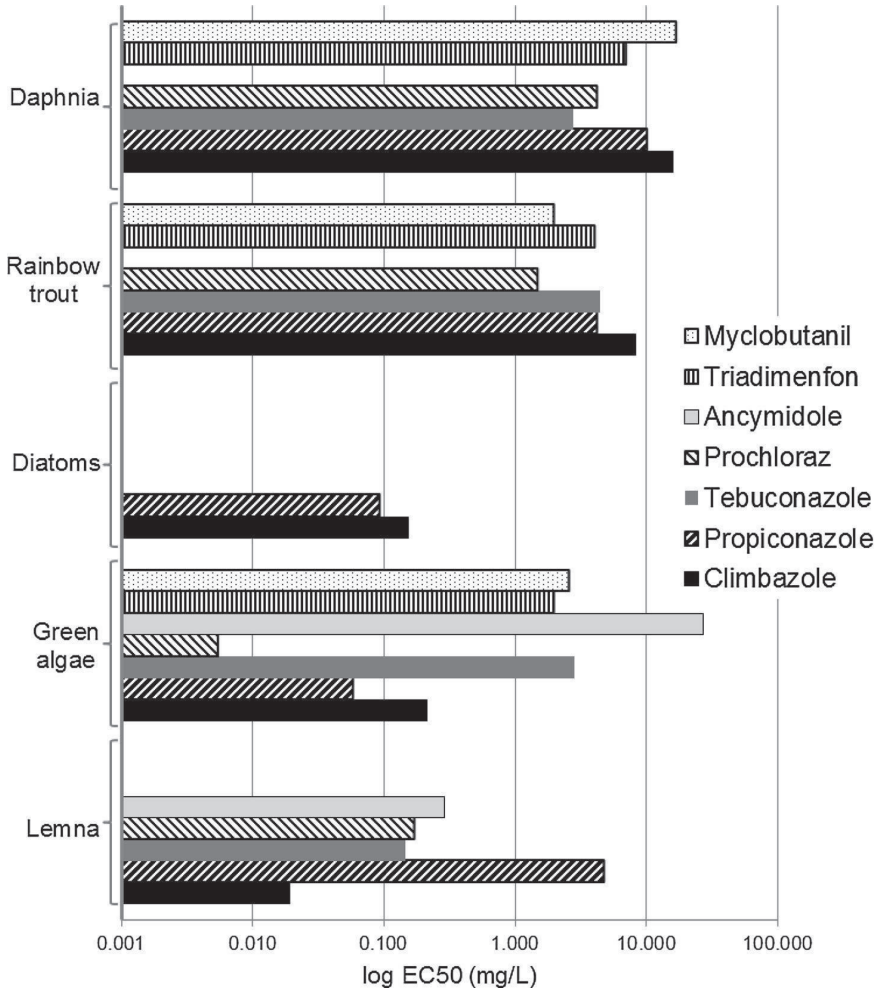


Figure 3. Comparison of toxicity of C14-demethylase inhibitor (DMI) fungicides toward aquatic species. The data are from regulatory databases, except for climbazole. Details on biotest endpoints: lemna, growth inhibition after 7 d; green algae, growth inhibition after 72 h to 96 h; diatoms, growth inhibition after 72 h to 96 h; daphnia, mortality of *Daphnia magna* after 48 h; rainbow trout, mortality after 48 h to 96 h.

factor in evaluating toxicity. According to the substance's pK_a value, the pH determines the ratio of neutral to charged molecules in the test solution. However, only the neutral molecule is assumed to be toxic because its lipophilic character promotes passive uptake and bioaccumulation in the cell [38], whereas membrane permeability and thus bioavailability of the corresponding charged molecule is thought to be relatively low [13]. Indeed, Rendal et al. [38] detected a positive correlation between the aquatic toxicity of a substance and the pH at which its neutral fraction prevails. Accordingly, climbazole, which is protonated at pH ranges below its pK_a, should be more toxic in test media with pH above 7.5. To test this hypothesis, we estimated the neutral molecule fraction in our aquatic biotests, keeping in mind that a direct comparison between different organisms tested in different media is problematic. However, no obvious correlation between climbazole toxicity and its speciation could be detected (Figure 1). It is all the more remarkable that with *Lemna*, the neutral molecule fraction in the medium was only 5% and yet it was affected at concentrations 1 order of magnitude lower than algae. Perhaps the cationic climbazole fraction to some extent contributed to the toxicity because as a macrophyte, *Lemna* possesses advanced uptake mechanisms compared with unicellular algae [35]. It is more likely, however, that the high toxicity to *Lemna* is related to the same species-specific mode of action as observed in the terrestrial plants, that is, the growth-retarding action. To conclude, the difference in climbazole toxicity to aquatic organisms is most probably species specific and the consequence of 2 target sites: the disturbance of membrane sterol composition and the particularly sensitive phytohormone balance.

Terrestrial toxicity of climbazole and influence of soil properties on phytotoxicity

From our results, climbazole can be regarded as nontoxic to enchytraeids and collembolas at concentrations up to 1000 mg/kg soil dry weight. Comparable studies on DMI fungicides are scarce but indicate an equally low toxicity to terrestrial microfauna and mesofauna. In *Eisenia fetida*, an oligochaete like the enchytraeids, propiconazole is estimated to be nontoxic, and tebuconazole and triadimenol are considered slightly harmful, with median lethal concentrations (14 d) of 1381 mg/kg soil dry weight and 781 mg/kg soil dry weight, respectively [36]. However, the protonation in soil of organic substances should also be considered [39], although this is

often neglected. Regarding our faunal biotests, theoretically more than 90% of climbazole was positively charged because of the acidic soil pH (6.0) and thus was probably unavailable for dermal uptake. Indeed, it has been pointed out for the weak base carbendazim that soil pH is a critical factor in determining its toxicity to earthworms [39].

Traditionally, guidelines on terrestrial biotests for pesticide assessment regulate the soil organic matter (SOM) content relatively strictly (peat content of the artificial soil should be 5% for test substances with log $K_{OW} > 2$ and 10% for log $K_{OW} \leq 2$) [16], as it is assumed that bioavailability and thus toxicity of hydrophobic substances is negatively related to SOM [40]. However, our results demonstrate that for the phytotoxicity of climbazole, the SOM is of minor relevance, while the soil pH plays a pivotal role (Table 4). These findings can probably be attributed to mainly 3 processes in relation to climbazole: its pH-dependent speciation, the SOM-dependent adsorption, and the plant-specific uptake mechnisms. At low soil pH ($pH < pK_a$ of 7.5), the protonated climbazole fraction prevails, while at high pH (>7.5), the uncharged fraction increases. Presumably, ionic binding of the cation to the overall negatively charged soil surface is stronger than nonspecific adsorption of the neutral molecule. Indeed, effects on biomass and shootlength of *B. napus* were up to 30% higher at soil pH 7.7 (~60% neutral molecule) compared with the acidic pH 5.5 (~1% neutral molecule), indicating that the neutral molecule possesses higher bioavailability and thus phytotoxicity. However, the neutral molecule with a calculated log K_{OW} of 3.33 can be classified as hydrophobic and therefore should strongly adsorb to the organic portion of the soil. However, even in soils where theoretically only 1% of climbazole was present in the neutral form, plant growth was greatly affected, independent of the peat content. This could be explained by the fact that uptake and exposure in higher plants, in contrast to animals, is not restricted to substances dissolved in the soil solution, but comprises active mobilization of both neutral molecules and ions in their rhizosphere, for example, via root exudates and mycorrhizae [41]. Moreover, owing to the very large surface area of fine roots, spatial exploitation of the soil is usually higher with plants than with soil fauna. Thus, SOM content might have reduced the climbazole concentration in the soil solution, but did not influence phytotoxicity because the cationic climbazole should also have been plant-available to a certain degree. Only for the endpoint seedling emergence did toxic effects of climbazole follow expectations, decreasing with increasing SOM. A reason for this observation may be that during germination, when uptake processes are still passive [41], exposure of the seeds is limited to the climbazole concentration dissolved in the soil solution, which is the SOM-dependent neutral molecule fraction.

In conclusion, it can be noted that within the pH range of 5.0 to 7.5, which is acceptable and common for tests according to the international plant test guidelines [22,26], toxic effects of climbazole can be substantially different because of the alteration of the ratio of its positive and neutral form. Similar dependencies are expected for other organic contaminants with pK_a values in the range of 5 to 9, as their physicochemical properties change significantly by protonation or deprotonation.

Environmental risks of climbazole

Climbazole is registered in the European Union with a manufacture and import volume of 100 tons/annum to 1000 tons/annum, which is the second highest tonnage band category (www.echa.europa.eu). However, detailed data on production volumes and predicted environmental concentrations are not available for climbazole, which hampers an environmental risk assessment. In an ongoing monitoring project, climbazole has been detected in the effluent of 2 German conventional WWTPs, with maximum concentrations of 0.110 μg/L [7]. The available data so far indicate that climbazole is introduced into surface waters with concentrations in WWTP effluents being approximately 2 orders of magnitude lower than those affecting *Lemna*, the most sensitive aquatic test organism identified in the present study. This might be an indication for a limited environmental risk of climbazole as an individual substance. However, environmental risks may be increased under simultaneous exposure with other substances. Particularly for mixtures containing DMI fungicides, additive or even more than additive effects have been reported [42,43].

CONCLUSIONS

The ecotoxicity of the cosmetic ingredient climbazole proved to be mostly similar to that of other DMI fungicides that are used as plant protection products or pharmaceuticals. Exceptionally high toxicity was found toward the aquatic macrophyte *Lemna* and terrestrial plants, with phenotypic symptoms in higher plants being similar to those caused by plant growth retardants. This supports the hypothesis that climbazole, in addition to acting as a DMI, specifically inhibits biosynthesis of the phytohormone gibberillin. Aquatic toxicity data for macrophytes are normally not required in regulatory risk assessment procedures for fungicides, which appears to be a substantial shortcoming considering the sensitivity of higher plants to antifungals such as climbazole. In addition, the present study demonstrates that for climbazole as an ionizable substance, phytotoxicity is less influenced by soil peat content and more by soil pH, a parameter that is not very closely regulated in soil standard test guidelines.

SUPPLEMENTAL DATA

Tables S1–S3. (94 KB PDF).

Acknowledgment—The authors thank J. Oehlmann for valuable discussions and R. Duering for soil characterization. The present study was financially supported by the German Federal Ministry of Education and Research through the project ELaN (FKZ: 033L025F).

REFERENCES

1. Ternes T, Knacker T, Oehlmann J. 2003. Körperpflegemittel in der aquatischen Umwelt. *Z Umweltchem Ökotox* 15:169–180.
2. Daughton CG, Ternes TA. 1999. Pharmaceuticals and personal care products in the environment: Agents of subtle change? *Environ Health Perspect* 107:907–938.
3. European Union. 2008. Commission Directive 2008/42/EC of 3 April 2008 last amending Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products. *Official Journal of the European Union L* 262.
4. Ternes TA, Joss A, Siegrist H. 2004. Scrutinizing pharmaceuticals and personal care products in wastewater treatment. *Environ Sci Technol* 38:392A–399A.
5. Boxall ABA, Rudd MA, Brooks BW, Caldwell DJ, Choi K, Hickmann S, Innes E, Ostapyk K, Staveley JP, Verslycke T, Ankley GT, Beazley KF, Belanger SE, Berninger JP, Carriquiriborde P, Coors A, DeLeo PC, Dyer SD, Ericson JF, Gagné F, Giesy JP, Gouin T, Hallstrom L, Karlsson MV, Larsson DGJ, Lazorchak JM, Mastrocco F, McLaughlin A, McMaster ME, Meyerhoff RD, Moore R, Parrott JL, Snape JR, Murray-Smith R, Servos MR, Sibley PK, Straub JO, Szabo ND, Topp E, Tetreault GR, Trudeau VL, Van Der Kraak G. 2012. Pharmaceuticals and personal care products in the environment: What are the big questions? *Environ Health Perspect* 120:1221–1229.

6. Brausch JM, Rand GM. 2011. A review of personal care products in the aquatic environment: Environmental concentrations and toxicity. *Chemosphere* 82:1518–1532.
7. Wick A, Fink G, Ternes TA. 2010. Comparison of electrospray ionization and atmospheric pressure chemical ionization for multi-residue analysis of biocides, UV-filters and benzothiazoles in aqueous matrices and activated sludge by liquid chromatography-tandem mass spectrometry. *J Chromatogr A* 1217:2088–2103.
8. Scientific Committee on Consumer Products. 2009. Opinion on climbazole. SCCP/1204/08., European Commission, Brussels, Belgium.
9. Perez-Rivera AA, Hu T, Aardema MJ, Nash JF. 2009. Evaluation of the genotoxicity of the imidazole antifungal climbazole: Comparison to published results for other azole compounds. *Mutat Res-Gen Tox En* 672:27–39.
10. Kobayashi Y, Suzuki M, Ohshiro N, Sunagawa T, Sasaki T, Oguro T, Tokuyama S, Yamamoto T, Yoshida T. 2002. Induction and inhibition of cytochrome P450 and drug-metabolizing enzymes by climbazole. *Biol Pharm Bull* 25:53–57.
11. Maertens JA. 2004. History of the development of azole derivatives. *Clin Microbiol Infec* 10:1–10.
12. Lamb D, Kelly D, Kelly S. 1999. Molecular aspects of azole antifungal action and resistance. *Drug Resist Update* 2:390–402.
13. Escher BI, Hunziker R, Schwarzenbach RP. 1999. Kinetic model to describe the intrinsic uncoupling activity of substituted phenols in energy transducing membranes. *Environ Sci Technol* 33:560–570.
14. Neuwoehner J, Escher BI. 2011. The pH-dependent toxicity of basic pharmaceuticals in the green algae *Scenedesmus vacuolatus* can be explained with a toxicokinetic ion-trapping model. *Aquat Toxicol* 101:266–275.
15. Organisation for Economic Co-operation and Development. 1984. Test No. 207: Earthworm, acute toxicity tests. *OECD Guidelines for the Testing of Chemicals*. Paris, France.
16. European and Mediterranean Plant Protection Organization. 2003. Environmental risk assessment scheme for plant protection products. *OEPP/EPPO Bulletin* 33:147–149.
17. Organisation for Economic Co-operation and Development. 2006. Test No. 221: *Lemna sp*. growth inhibition test. *OECD Guidelines for the Testing of Chemicals*. Paris, France.
18. Organisation for Economic Co-operation and Development. 2006. Test No. 201: Freshwater alga and cyanobacteria, growth inhibition test. *OECD Guidelines for the Testing of Chemicals*. Paris, France.
19. Organisation for Economic Co-operation and Development. 2006. OECD draft proposal for a new guideline: Fish embryo toxicity (FET) test. Paris, France.
20. Organisation for Economic Co-operation and Development. 1992. Test No. 203: Fish, acute toxicity test. *OECD Guidelines for the Testing of Chemicals*. Paris, France.
21. Organisation for Economic Co-operation and Development. 2004. OECD Test No. 202: *Daphnia sp*., acute immobilisation test. *OECD Guidelines for the Testing of Chemicals*. Paris, France.
22. International Organization for Standardization. 2012. Soil quality—Determination of the effects of pollutants on soil flora. Part 2: Effects of chemicals on the emergence and growth of higher plants. ISO 11269-2. Geneva, Switzerland
23. Organisation for Economic Co-operation and Development. 2004. Test No. 220: Enchytraeid reproduction test. *OECD Guidelines for the Testing of Chemicals*. Paris, France.
24. Organisation for Economic Co-operation and Development. 2009. Test No. 232: Collembolan reproduction test in soil. *OECD Guidelines for the Testing of Chemicals*. Paris, France.
25. International Organization for Standardization. 2008. Water quality—Determination of the inhibition of dehydrogenase activity of *Arthrobacter globiformis*: Solid contact test using the redox dye resazurine. ISO 10871. Geneva, Switzerland.
26. Organisation for Economic Co-operation and Development. 2006. Test No. 208: Terrestrial plant test: Seedling emergence and seedling growth test. *OECD Guidelines for the Testing of Chemicals*. Paris, France.
27. R Development Core Team. 2010. *R: A Language and Environment for Statistical Computing*. Ver 2.11.1. R Foundation for Statistical Computing, Vienna, Austria.
28. Ritz C, Streibig JC. 2005. Bioassay analysis using R. *J Stat Softw* 12: 1–22.
29. Levine TR, Hullett CR. 2002. Eta squared, partial eta squared, and misreporting of effect size in communication research. *Hum Commun Res* 28:612–625.
30. Stenersen J. 2004. *Chemical Pesticides: Mode of Action and Toxicology*. CRC Press, Boca Raton, Florida.
31. Maltby L, Brock TCM, Van den Brink PJ. 2009. Fungicide risk assessment for aquatic ecosystems: Importance of interspecific variation, toxic mode of action, and exposure regime. *Environ Sci Technol* 43:7556–7563.
32. Lepesheva GI, Waterman MR. 2007. Sterol 14α-demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms. *BBA Gen Subjects* 1770:467–477.
33. Rademacher W. 2000. Growth retardants: Effects on gibberellin biosynthesis and other metabolic pathways. *Annu Rev Plant Physiol* 51:501–531.
34. Thies F, Horst Grimme L. 1996. Effects of cytochrome P450-interacting plant growth retardants, fungicides and related compounds on cell development and phase-I biotransformation capacity of unicellular photoautotrophic green algae. *Pestic Sci* 47:337–346.
35. Inada S, Tominaga M, Shimmen T. 2000. Regulation of root growth by gibberellin in *Lemna minor*. *Plant Cell Physiol* 41:657–665.
36. European Commission. 2013. European Union Pesticides Database. [cited 24 January 2013]. Available from: http://ec.europa.eu/sanco_pesticides
37. US Environmental Protection Agency. 2013. [cited 24 January 2013]. Available from: http://www.epa.gov/pesticides/.
38. Rendal C, Kusk KO, Trapp S. 2011. Optimal choice of pH for toxicity and bioaccumulation studies of ionizing organic chemicals. *Environ Toxicol Chem* 30:2395–2406.
39. Liu K, Pan X, Han Y, Tang F, Yu Y. 2012. Estimating the toxicity of the weak base carbendazim to the earthworm (*Eisenia fetida*) using in situ pore water concentrations in different soils. *Sci Total Environ* 438: 26–32.
40. Van Gestel CAM. 1992. The influence of soil characteristics on the toxicity of chemicals for earthworms: A review. In Greig-Smith PW, Becker H, Edwards PI, Heimbach F, eds *Ecotoxicology of Earthworms*. Intercept, Andover, UK, pp 45–54.
41. Buchanan B, Gruissem W, Jones R. 2002. *Biochemistry & Molecular Biology of Plants*. John Wiley & Sons, Hoboken, NJ, USA.
42. Norgaard KB, Cedergreen N. 2010. Pesticide cocktails can interact synergistically on aquatic crustaceans. *Environ Sci Pollut Res* 17: 957–967.
43. Coors A, Frische T. 2011. Predicting the aquatic toxicity of commercial pesticide mixtures. *Environ Sci Eur* 23:22.
44. Schwarzenbach RP, Gschwend PM, Imboden DM. 2003. *Environmental Organic Chemistry*. Wiley-Interscience, Hoboken, NJ, USA.

SUPPLEMENTARY DATA

Table S1: Details of the conducted biotest: Test organisms, test duration, test medium, replication and guidelines used. Identical conditions applied to cultures and biotests.

Test organism and taxonomic group	Strain	Origin	Test medium or substrate	PH	Feeding regime	Temp. regime [°C]	Light regime	Volume per replicate [mL or g]	No. of replicates	Test concentrations [mg/L or mg/kg]
L. minor Macrophyta, Araceae	clone St, Landolt number 9441	in-house batch culture, from Friedrich-Schiller University Jena, K.-J. Appenroth	Steinberg medium modified acc. R. Altenburger (annex OECD 221)	5.6 / 6.9; 6.2	---	24 ± 2	continuous PAR of 85 - 135 µE/m2*s (mean 113)	200	3 / 6 (4 colonies)	0.008 – 0.200
N. pelliculosa Phytoplankton, Bacillariophyceae	1050-3 SAG	in-house batch culture, from SAG University Göttingen	AAP medium (annex OECD 201)	7.5 / 9.6; 8.3	---	21 - 24	permanent cool-white fluorescent, mean 98 µE/m2*s	100	3 / 6 ($4*10^4$ cells/mL)	0.008 – 0.200
P. subcapitata Phytoplankton, Chlorophyta	61.81 SAG	in-house culture, from SAG University Göttingen	medium acc. to Kuhl and Lorenzen (1964)	6.0 / 6.1; 6.0	---	21 - 24	permanent cool-white fluorescent, mean 89 µE/m2*s	100	3 / 6 ($0.5*10^4$ cells/mL)	0.150 – 2.40
D. rerio Chordata, Cyprinidae		in-house culture	reconstituted water acc. to OECD 203, supplemented with 0.1% artificial seawater (28 g/L of Tropic Marin sea salt, Dr. Biener)	7.5 / 7.4; 7.5	brood fish: trice per day (1 time live *Artemia spec.*, 2 times dry feed), no feeding during test	26 ± 1	permanent light, mean 300 lux	100	4 (10)	0.316 – 31.6
D. magna Arthropoda, Crustacea	STRAUS, clone 5	in-house culture, from Dr. Ensenbach, AVENTIS, Frankfurt / Main	Elendt medium M4 (annex OECD 202)	7.8 / 7.8; 7.7	culture: trice per week (green algae)	22 ± 1	16:8 light:dark cycle with mean 450 lux	150	4 (5)	1.88 – 30.0

A. sativa higher plants, Monocotyledonae	ST. Champion	Saaten-Union GmbH, Isernhagen HB	LUFA 2.3 and art. soil	6.8 / 7.1; 6.9 and 6.0 / 5.7; 5.8	commercial liquid fertiliser (Substral) at 50 mL per pot once	22 ± 10	16:8 light:dark cycle with high pressure metal halide lamps (Master HPI-T PLUS, Philips GmbH, Hamburg, Germany), mean 230 µE/m2*s	300	4 (5 plants)	3.16 – 316
B. napus higher plants, Dicotyledonae	Liforum	LUFA Speyer	LUFA 2.3 and art. soil	6.8 / 6.8; 6.7 and 6.0 / 5.7; 5.9	commercial liquid fertiliser (Substral) at 50 mL per pot once	22 ± 10	as for A. sativa	600	4 (5 plants)	3.16 – 316
A. globiformis Bacteria, Actinobacteria	DSM No. 20124	lyophilisat of culture from German Collection of Microorganisms and Cell Cultures	quartz sand	6.8 / 6.8; 6.8	lyophilisat resuspended in 20 mL nutrient solution B (acc. ISO draft)	30 ± 2	dark climate chamber	0.6	4 (0.4 mL cell suspension)	1 - 1000
E. bigeminus Annelida, Oligochaeta		in-house culture	art. soil	6.1 / 6.0; 6.0	Oatmeal at 50 mg per test vessel at day 0 and 25 mg at day 7, 14 and 21	20 ± 2	16:8 light:dark cycle with mean 420 lux	20	4 (10)	1 - 1000
F. candida Arthropoda, Tracheata		in-house culture, from Freie Universität Berlin, R. Achazi	art. soil	6.1 / 6.1; 6.1	granulated dried baker's yeast, per test vessel 20 mg at day 0 and 10 mg at day 14	20 ± 2	16:8 light:dark cycle with mean 417 lux	30	4 (10)	1 - 1000

d = days, h = hours, min = minutes, w = weeks, mod. = modified, acc. = according to; art. soil = artificial soil according to OECD 207 with 5% peat. PH in control at test start / test end; mean pH in treatments (over test start and end), PAR = photosynthetically active radiation, number of replicates per control / per treatment, (no. of individuals per replicate).

Table S2: Validity criteria requested by the guidelines and compliance of the biotests

Test organism	Validity criteria for the controls		
L. minor	frond number doubling time < 2.5 days: OK (1.8)		
N. pelliculosa	increase of cell number between 0 and 72 h > factor 16: OK (56)	mean section-by-section growth rate < 35%: OK (21%)	coefficient of variation of mean specific growth rate from 0 to 72 h < 10%: OK (2%)
P. subcapitata	increase of cell number between 0 and 72 h > factor 16: OK (286)	mean section-by-section growth rate < 35%: OK (21%)	coefficient of variation of mean specific growth rate from 0 to 72 h < 7%: OK (2%)
D. rerio	survival of embryos after 48 h ≥ 90%: OK (97%)[a]		
D. magna	immobilised daphnids after 48 h < 10%: OK (0%)	dissolved oxygen concentration after 48 h ≥ 3 mg/L: OK (≥ 9.6 mg/L)	
A. sativa	≥ 70% emergence in each pot: OK (≥ 80%)		
B. napus	≥ 70% emergence in each pot: OK (≥ 80%)		
A. globiformis	mean fluorescence increase from 0 to 60 min > factor 5: OK (61)	inhibition by reference substance BAC (CAS 122-18-9) between 30 and 80%: too high (98%)	
E. bigeminus	adult mortality ≤ 20%: OK (0%)	mean number of juveniles ≥ 25: OK (282)	coefficient of variation of number of juveniles ≤ 50%: OK (4%)
F. candida	adult mortality ≤ 20%: OK (15%)	mean number of juveniles ≥ 100: OK (364)	coefficient of variation of number of juveniles ≤ 30%: OK (16%)

[a] Criterion of ISO draft 15088 (2009), no validity criteria defined in OECD.

Table S3: Composition of LUFA 2.3 and the artificial soil used in the terrestrial dose-response tests

LUFA 2.3		Artificial soil according to OECD 207	
Organic carbon in %	0.94 ± 0.10	Peat in % [b]	5.0
Nitrogen in % N	0.08 ± 0.02	Kaolin clay in % [c]	20
Particles < 0.02 mm in %	21.6 ± 3.3	Quartz sand in % [d]	75
Cation Exchange Capacity (meq/100 g)	10.7 ± 1.4	Calcium carbonate in % [e]	0.2
Water Holding Capacity (g/100 g)	35.6 ± 3.0	Water Holding Capacity (g/100 g)	55.6
PH-value [a]	6.8 ± 0.2	PH-value	6.0
Soil Type (USDA)	sandy loam		
Soil Type (German DIN)	silty sand (uS)		
Weight per Volume (g/1000 mL)	1295 ± 30		

[a] Determined in 0.01 M $CaCl_2$ solution; [b] Sphagnum peat, Floragard GmbH, air dried and sieved to 4 mm; [c] Amber Kaolinwerke Eduard Kick GmbH & Co.KG; [d] F36, Quarzwerke GmbH, particle size: > 50% 50 to 200 µm; [e] $CaCO_3$, 99%, Carl Roth GmbH & Co.KG.

A.1.2 Phytotoxicity of wastewater-born micropollutants – characterisation of three antimycotics and a cationic surfactant

Richter E, Roller E, Kunkel U, Ternes TA, Coors A: Environmental Pollution.

(under revision, accepted with minor revisions 10 Sep 2015)

Author contributions:

Initials: Elisabeth Richter (ER), Elias Roller (ERO), Uwe Kunkel (UK), Thomas A Ternes (TT), Anja Coors (AC).

Study design and planning:

ER: 70%; AC: 30%.

Performance of the experiments and analyses:

ER: 20%, biological tests; ERO: 70%, biological tests; UK: 10%, chemical analysis.

Data assembling and preparing of figures:

ER: 60%, evaluation and preparation of graphs of biological and chemical results; ERO: 30%, evaluation of biological results and preparation of pictures, UK: 10%, data assembling of chemical results.

Data analysis and interpretation:

ER: 50%, analysis and interpretation of biological and chemical data; ERO: 5%, AC: 25%, UK: 10%, TT: 10%, discussion and further ideas for analysing and interpreting biological and chemical data.

Introduction, results and discussion:

ER: 60%; AC: 20%, UK, TT: 10%.

Phytotoxicity of wastewater-born micropollutants – characterisation of three antimycotics and a cationic surfactant

Elisabeth Richter [a, b], Elias Roller [a], Uwe Kunkel [c], Thomas A. Ternes [c], Anja Coors [a]

[a] ECT Oekotoxikologie GmbH, Böttgerstr. 2-14, 65439 Flörsheim / Main, Germany

[b] Department Aquatic Ecotoxicology, Goethe University Frankfurt am Main, Max-von-Laue-Str. 13, 60438 Frankfurt / Main, Germany

[c] Federal Institute of Hydrology (BfG), Am Mainzer Tor 1, 56068 Koblenz, Germany

Corresponding author: Elisabeth Richter, Böttgerstr. 2-14, 65439 Flörsheim / Main, Germany, telephone: +49 6145 9564 30, fax: +49 6145 9564 99, email: e.richter@ect.de

Key Words: Personal care products, pharmaceuticals, fungicides, azole, quaternary ammonium compound.

Abstract

Sewage sludge applied to soil may be a valuable fertiliser but can also introduce poorly degradable and highly adsorptive wastewater-born residues of pharmaceuticals and personal care products (PPCPs) to the soil, posing a potential risk to the receiving environment. Three azole antimycotics (climbazole, ketoconazole and fluconazole), and one quaternary ammonium compound (benzyldimethyldodecylammonium chloride, BDDA) that are frequently detected in municipal sewage sludge were therefore characterised in their toxicity toward terrestrial (*Brassica napus*) and aquatic (*Lemna minor*) plants. Fluconazole and climbazole showed the greatest toxicity to *B. napus*, while toxicity of ketoconazole and BDDA was by one to two orders of magnitude lower. Sludge amendment to soil at an agriculturally realistic rate of 5 t/ha significantly reduced the bioconcentration of BDDA in *B. napus* shoots, although not significantly reducing phytotoxicity. Ketoconazole, fluconazole and BDDA proved to be very toxic to *L. minor* with median effective concentrations ranging from 55.7 µg/L to 969 µg/L. In aquatic as well as terrestrial plants, the investigated azoles exhibited growth-retarding symptoms presumably related to an interference with phytohormone synthesis as known for structurally similar fungicides used in agriculture. While all four substances exhibited considerable phytotoxicity, the effective concentrations were at least one order of magnitude higher than concentrations measured in sewage sludge and effluent. Based on preliminary hazard quotients, BDDA and climbazole appeared to be of greater environmental concern than the two pharmaceuticals fluconazole and ketoconazole.

Introduction

The safe and sustainable disposal of sewage sludge is an issue inherently related to wastewater treatment and the various options are discussed controversially. On the one hand, sewage sludge is continously produced in wastewater treatment plants (WWTPs) and contains valuable nutrients and organic matter (Cogger *et al.* 2006); hence, a re-use in agriculture appears reasonable and is practised in many countries (Beecher *et al.*, 2008). On the other hand, sewage sludge contains a variety of wastewater-born pollutants, especially those that are poorly degradable and sorptive, with often unknown environmental effects (Clarke and Smith, 2011).

Therefore, sludge application to land may transfer wastewater-born pollutants to soil and thus pose a risk to agricultural soil quality (Schowanek, 2004, Eriksson *et al.*, 2008, Prosser *et al.*, 2014). Less sorptive substances may also desorb from land-applied sludge, reach adjacent surface waters via particle-bound run-off (Topp *et al.*, 2008) or be released to groundwater via irrigation with treated wastewater (Richter *et al.*, 2015). Therefore, assessing the environmental risk of wastewater-born pollutants should take into account both terrestrial and aquatic organisms (Clarke and Smith, 2011, Langdon *et al.*, 2010, Richter *et al.,* 2015). Ecotoxicity data are particularly scarce for many sludge-associated micropollutants such as pharmaceuticals and ingredients of personal care products (PPCPs) (Topp *et al.*, 2008, Clarke and Smith, 2011, Prosser *et al.*, 2014).

One group of micropollutants frequently detected in wastewater are antimycotics containing an azole moiety (Garcia-Valcarcel *et al.*, 2012, Peng *et al.*, 2012, Chen *et al.* 2013). Azoles are applied as fungicides in agriculture, but they are also used as active ingredients in cosmetics and pharmaceuticals. As demethylase inhibitors (DMI) azoles prevent the biosynthesis of ergosterol by inhibiting the cytochrome P450-dependent enzyme lanosterol 14-α-demethylase (CYP51) (Lepesheva and Waterman, 2007). However, many of them are known to also specifically interact with the hormonal balance of animals or plants (Rademacher, 2000).

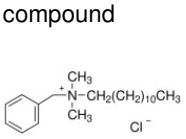
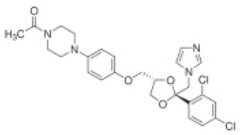
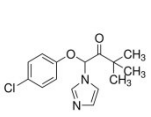
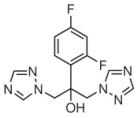
Quaternary ammonia compounds (QACs) represent another micropollutant group detected at high concentrations in sludge (Ferk *et al.*, 2007, Braguglia *et al*., 2014). QACs are used in numerous consumer products such as detergents and PPCPs (Uhl *et al.*, 2005) and in great quantities in the food and health care industries for cleaning and disinfection (Gerba, 2015). As cationic surfactants they disturb membranes but also interact with intracellular targets, exhibit genotoxicity or interact with the phytohormones of higher plants (Rademacher, 2000, Zhang *et al.*, 2011, Gerba, 2015). Some QACs are therefore applied as plant growth regulators (Rademacher, 2000, Miliuviene 2003).

From these two groups, four model substances were selected to represent various fields of application and different physicochemical properties with regard to their hydrophobicity and pH-dependent speciation (see Table 1). Ketoconazole, climbazole and fluconazole were selected to

represent azoles that are contained in systemic antimycotics, anti-dandruff shampoos and skin creams, respectively. They have been detected in sewage sludge at concentrations of 50-4450 µg/kg dry weight (d.w.) (Garcia-Valcarcel *et al.*, 2011, Wick *et al.*, 2010). As a QAC, BDDA (benzyldimethyldodecylammonium chloride) was selected which is detected at high concentrations in both effluent (up to 0.5 µg/L) and sewage sludge (up to 25 mg/kg d.w., Martinez-Carballo *et al.*, 2007, Chen *et al.*, 2014).

Land application of sewage sludge can alter the chemical and physical properties of the soil, e.g. the organic matter content that can consequently affect the fate and effects of micropollutants in soil (Reid *et al.*, 2000, Wu *et al.*, 2009). Hence, testing their effects on soil organisms in the presence of a sludge matrix has been proposed as a more realistic approach compared to the commonly applied standard ecotoxicity tests with natural or artificial soil (Schowanek *et al.*, 2004, Prosser *et al.*, 2014).

Table 1: Characteristics of the four test substances.

Name	**BDDA**	**Ketoconazole**	**Climbazole**	**Fluconazole**
CAS No.	139-07-1	65277-42-1	38083-17-9	86386–73-4
Molecular weight [g/mol]	340 (cation: 304.5)	531	293	306
Log K_{ow} [1]	2.93	4.35	3.76	0.5
Log K_{oc} [1]	5.43	4.26	3.08	3.59
pK_a	not applicable	6.51 / 2.94 [2]	7.5 [3]	2.5; 2.9; 11.0 [4]
Chemical class	quaternary ammonium compound	imidazole	imidazole	triazole
Structure				
Supplier of test item (purity)	Sigma-Aldrich Germany (99.0%)	TCI GmbH Germany (99.5%)	TCI GmbH Germany (99.9%)	TCI GmbH Germany (99.6%)

[1] EPISUITE, KOWWIN v1.68 and KOCWIN v2.0, MCI estimates; [2] Skiba et al., 2000; [3] Wick et al., 2010; [4] Correa et al., 2012. BDDA = benzyldimethyldodecylammonium chloride.

The aim of the present study was therefore to i) determine the phytotoxicity of four wastewater-born PPCPs for which specific toxicity toward plants was assumed based on previous reports on structurally related substances (Rademacher, 2000, Miliuviene 2003) using the aquatic plant *Lemna*

minor and the terrestrial plant *Brassica napus*, and to ii) determine to which extent the presence of co-applied sludge would modify terrestrial phytotoxicity based on studies reporting an reduced pollutant availability (Wu *et al.*, 2009, Holling *et al.*, 2012, Peña *et al.*, 2014, Prosser *et al.*, 2014). Considering their log K_{ow} and pK_a (Table 1) the substances cover a range from hydrophilic (fluconazole), to medium and strongly hydrophobic properties (climbazole and ketoconazole, respectively). Based on the scheme given by Schaffer and Licha (2015), they should be uncharged (fluconazole), predominantly (climbazole and ketoconazole) or permanently cationic (BDDA) at the test medium or soil pH of about 7. As hydrophobicity drives partitioning to organic matter and cationic species strongly interact with negatively charged soil surfaces (Schaffer and Licha, 2015), the influence of sludge-amendment on phytotoxicity was expected to be greatest for ketoconazole and BDDA.

Materials and methods

Test substances:

BDDA (as "benzododecinium chloride") is registered as existing biocide (EC, 2014). In literature, also the synonym BAC-C12 is used (Clara *et al.*, 2007). All BDDA concentrations in the present study refer to the cation (i.e. without the chloride moiety) as it is permanently dissociated in liquid and solid matrices and only the cation can be analytically determined. Fluconazole is used as antimycotic agent in the systemic and topical treatment of patients infected with various *Candida* species (EMA/888361/2011). Ketoconazole is a broad-spectrum antimycotic agent that has recently been suspended for oral but not for topical administration in the EU (EMA/CHMP/534845/2014). It is additionally registered as preservative in topical cosmetics and personal care products (with maximum concentration of 1% in rinse-off and leave-on products and of 2% in licensed anti-dandruff shampoos). Climbazole is likewise registered as preservative in topical cosmetics and personal care products (with maximum concentration of 0.5% in rinse-off and leave-on products and of 2% in anti-dandruff shampoos) and is registered under REACH with a production volume of 10–100 t/a (http://echa.europa.eu). Key characteristics of the test substances are compiled in Table 1.

Biotests with *Lemna minor:*

The *L. minor* growth inhibition tests were conducted according to the OECD guideline 221 (OECD, 2006a) in modified Steinberg medium at 24°C ± 2°C under continuous photosynthetically active radiation (PAR) of 85–135 µE/(m^2s) (mean: 113 µE/(m^2s)) using plants cultured under the same conditions. At test start, mean pH in the controls and test solutions was 5.6 ± 0.1. Due to plant

growth-related uptake of bicarbonate, the pH increased during the exposure to 6.5 ± 0.4 (controls), 6.1 ± 0.3 (BDDA), 6.6 ± 0.3 (fluconazole) and 6.8 ± 0.3 (ketoconazole) (details see supplements Table S1). For climbazole, data on toxicity to *L. minor* had been generated previously under similar conditions (Richter *et al.*, 2013).

Fluconazole and BDDA were directly dissolved in test medium, while for ketoconazole a stock solution was prepared in acetone (final concentration in treatments and solvent control 0.01% (v/v)). For each substance, five concentrations were tested (see supplements Table S2). Tests were performed in glass vessels containing 200 ± 5 mL test solution and four *Lemna* colonies each (i.e. 12 fronds) covered with glass lids, using three replicates per treatment/concentration and six for medium controls.

Fluconazole and ketoconazole were tested in a static design over seven days. Due to dissipation observed in pre-tests, BDDA was tested in a semi-static design with *Lemna* colonies being transferred to freshly prepared test solution at day 2 and 5. Fronds were counted, visually inspected and test vessels were randomized twice (day 2 and 5) in all tests. The pH was measured in each treatment at test start and test end (and day 2 and 5 for BDDA). At test end (day 7), frond number and frond dry weight (biomass) were determined and their yield (increase from day 0 to day 7) and growth rate (average specific growth rate per day μ_{i-j}) were calculated according to equation 1

$$\mu_{i-j} = \frac{\ln(N_j) - \ln(N_i)}{t} \quad (1)$$

with N_i and N_j as the measurement variable in the test or control vessel at time i and j, respectively, and t as the time period from i to j.

Biotests with *Brassica napus:*

The seedling emergence and growth inhibition test with the plant *B. napus* was conducted according to the OECD guideline 208 (OECD, 2006b). A sandy loam, LUFA 2.3 (LUFA, Speyer, Germany), was used as test substrate (soil pH of 6.8 ± 0.2, organic carbon content (C_{org}) of 1.0% ± 0.1%, cation exchange capacity of 10.7 mEq/100 g, and maximum water holding capacity (WHC_{max}) of 37%). Each substance was tested in two treatments: without ("-bios") and with amendment of biosolids ("+bios") to the soil. The term biosolids is used in North America for stabilized sewage sludge complying with regulatory quality requirements for application as agricultural fertilizer. The biosolids were anaerobically digested, thermally stabilized municipal sewage sludge commercially available from Halton Company, Ontario, Canada, and produced for land application in Ontario (sampled May 28, 2013 and supplied by Agriculture and Agri-Food Canada, Ontario, Canada) with a C_{org} of 33.9% ± 0.1%. Based on the maximum application rate given by the German Sewage Sludge

Directive (Klärschlammverordnung, 1992), an amendment of 5 t/ha was selected as realistic scenario, which transfers to 3.8 g sludge d.w./kg soil d.w. assuming a soil incorporation depth of 10 cm and a soil bulk density of 1.3 g/cm^3.

The test substances were applied to soil as aqueous test solution (BDDA, fluconazole) or as ground mixture with quartz sand (ketoconazole, climbazole) at five concentrations, see supplements Table S2) and soil moisture was adjusted to approximately 35% of WHC_{max}. Afterwards, each soil batch (control and test concentrations) was divided into two parts of which one was left as such (-bios) and the other part was mixed with biosolids (+bios). By this addition, the test concentrations for all four substances decreased by less than 0.4%. Each soil batch was then distributed to four replicate pots (polypropylen pots, 11 cm diameter, 9 cm height, approximately 0.3 kg soil d.w.). In each pot five *B. napus* seeds (cultivar Liforum, LUFA, Speyer, Germany) were sown. The tests were performed in a climate controlled room at an air humidity of 70% ± 25%, 22°C ± 5°C temperature and a 16:8 light to dark cycle using high pressure metal halide lamps (Master HPI-T PLUS, Philips GmbH, Hamburg, Germany) with mean PAR of 230-20 µE/m^2*s. Pots were bottom-watered as necessary with deionized water, additionally supplied with liquid fertilizer once per week and randomized three times per week. Day 0 is defined as the day when 50% of seedlings in the respective control had emerged, which in the present study was three days after the test start. The morphological status of the plants was recorded on day 7 and day 16. As endpoints, the emergence rate on day 7, the survival rate until day 16, shoot length (in mm), and shoot fresh weight (biomass, in g) of the plants at test end (day 16) were evaluated.

To determine the uptake of BDDA into plants, shoots were sampled at test end, combined per replicate (i.e. pot), dried at 60°C for 13 h, the sum shoot d.w. per pot was determined, and plants were stored dry in the dark until analysis. After removing the roots, the soil of each pot was sampled and stored frozen to determine the BDDA concentrations in soil (for details see supplements).

Chemical analysis:

In the *L. minor* tests with fluconazole, ketoconazole and climbazole (Richter *et al.*, 2013), samples from all test concentration levels were taken at day 0 (fresh / initial) and day 7 (aged). In the test with BDDA, samples were taken from the lowest, medium and highest test concentration at day 0 (fresh), 2 (fresh and aged), 5 (fresh and aged) and 7 (aged). All samples were stored frozen until chemical analysis. If measured concentrations in aged solutions deviated by more than 20% from measured concentrations in fresh solutions, overall recovery was calculated as geometric mean of the recoveries over time for each test concentration level and the overall recovery as arithmetic mean of these geometric means.

Details on the analytical methods for the determination of ketoconazole, BDDA and fluconazole in water, the extraction of BDDA from soil and plants and the HPLC-MS/MS measurements are given in the supplements.

Statistical analyses:

Results of the biotests were analysed in the free software R version 3.2.1 using the package "drc" (R Development Core Team, 2015; Ritz and Streibig, 2005). To enable comparisons between different biotests and substances, all observed response variables were converted to percent inhibition relating to the mean response in the respective control (i.e., with or without biosolids, if applicable). These proportional effects were fitted using a two parameter log-logistic function (equation 2) with the limits fixed at 0 and 100, the parameter *b* relating to the slope of the curve and the median effective concentration (EC_{50}) being directly fitted as model parameter:

$$f(x) = \frac{1}{1 + \exp(b(\log(x) - \log(EC_{50})))} \qquad (2)$$

The EC_{10}, EC_{20} and EC_{50} (concentration with 10%, 20%, or 50% effect, respectively) and their 95% confidence intervals (CI) were estimated by the ED function using the delta method in the "drc" package. All concentration-response modelling was based on nominal test concentrations. Thereafter, EC_x values for *L. minor* were corrected based on overall recovery of measured concentrations.

Controls (-bios and +bios) were compared by Fisher`s Exact Binomial test with Bonferroni correction (emergence and survival data) or Student's t-test (shoot length and biomass data). No observed effect concentrations (NOECs) were determined at $\alpha = 0.05$ (one-sided) for emergence and survival of seedlings of *B. napus* applying the Fisher`s Exact Binomial test with Bonferroni correction and for all other endpoints applying the William's t-test (homogeneous variances) or the Welch's t-test (heterogeneous variances) at $\alpha = 0.05$ (one-sided) using ToxRat version 2.10 (ToxRat Solutions GmbH, Alsdorf, Germany).

The influence of sludge amendment on phytotoxicity was assessed by comparing the parameters of the two concentration-response curves (+bios and –bios) using the ratio test (Wheeler *et al.* 2006) in the function "compParm" of the drc package in R.

The influence of sludge amendment on BDDA shoot concentrations in *B. napus* was assessed in a one-way ANOVA for the factor 'biosolids'. Additionally, the BDDA bioconcentration factor ($BCF_{B.\ napus\ \text{shoot}}$, analogously to Peña *et al.*, 2014) was calculated for each pot as the ratio of average shoot concentration to the nominal soil concentration. This $BCF_{B.\ napus\ \text{shoot}}$ was assessed in a two-way

ANOVA for the factors 'biosolids' and 'BDDA concentration'. Normal distribution of errors and variance homogeneity were verified by Shapiro-Wilk's and Bartlett's test, respectively.

Results and discussion

Aquatic phytotoxicity:

Chemical analysis of the test solutions confirmed a generally good recovery for fluconazole (Table 2 and supplements Table S3), but poor recovery for BDDA and ketoconazole with measured concentrations in aged solutions reaching less than 20% of initial concentrations measured in fresh solutions.

Table 2: Mean recovery of test concentrations in *L. minor* bioassays in initial and aged test solutions and overall recovery as mean of geometric mean measured concentrations per concentration level.

Substance	Recovery initial to nominal [%]	Recovery aged to measured initial [%]	Overall mean recovery [%]
BDDA	68.2	64.4	43.6
Ketoconazole	45.0	8.54	11.8
Fluconazole	121	102	121

Test solutions analysed at test start (initial) and end (aged) in C1-C5 (fluconazole, ketoconazole) or at each renewal (day 3 and 5) in C1, C3 and C5 (BDDA, semi-static test design). BDDA = benzyldimethyldodecylammonium (cation).

Therefore, their overall recovery was calculated (see chapter 2.4) and effective concentrations are additionally provided as recovery-corrected values (Table 3). Sorption to glassware probably caused the low recovery of initial (fresh) test concentrations, while degradation may have contributed to the low recovery in aged test solutions of BDDA (i.e. after 2-3 days of exposure) and of ketoconazole (after 7 days) which is supported by literature on their degradability in WWTPs (Zhang *et al.*, 2011, Peng *et al.*, 2012). Experimental data for climbazole toxicity to *L. minor* were reported previously (Richter *et al.*, 2013, with overall mean recovery of 70.5%). These data were re-calculated in the present study (with <2% difference to previously reported EC_x) to ensure a consistent data analysis for all four test substances.

Table 3: Effective concentrations (EC_x with 95% confidence intervals, CI) and no observed effect concentrations (NOECs) of test substances determined for *L. minor* based on nominal concentrations (in mg/L) and corrected for overall recovery.

EC_x (95% CI)	**BDDA**	**Ketoconazole**	**Climbazole**	**Fluconazole**
		Yield FN		
NOEC	0.0896	0.100	0.0031	0.300
EC_{10}	0.154	0.178	0.0078	0.371
	(0.112 - 0.196)	(0.115-0.241)	(0.0062-0.0094)	(0.301-0.442)
EC_{50}	0.686	0.472	0.0195	0.969
	(0.597 - 0.775)	(0.406-0.539)	(0.0171-0.0219)	(0.886-1.05)
		MGR FN		
NOEC	0.269	0.190	0.0031	0.300
EC_{10}	7.09*	0.286	0.0113	0.473
	(0.00 - 28.3)	(0.227-0.345)	(0.0079-0.0147)	(0.369-0.577)
EC_{50}	7.22	0.743	0.0341	1.85
	(0.00 - 28.8)	(0.678-0.808)	(0.0341-0.0382)	(1.66-2.04)
Overall recovery [%]	43.6	11.8	70.5	121
		Yield FN recovery corrected		
EC_{10}	0.0670	0.0210	0.0055	
EC_{50}	0.299	0.0557	0.0138	
		MGR FN recovery corrected		
EC_{10}	3.09	0.0337	0.0080	
EC_{50}	3.15	0.0877	0.0240	

BDDA = benzyldimethyldodecylammonium (cation). FN = frond number; MGR = mean growth rate. *extrapolated beyond tested concentrations; values for climbazole are re-calculated based on proportional responses from Richter et al. 2013.

All bioassays with *L. minor* met the validity criteria of the test guideline (supplements Table S4). The estimated EC_x and NOECs are summarized in Table 3 for frond number (further details see supplements Table S5). EC_{50} values were below 1 mg/L for all four test substances regarding yield frond number (YFN) but only for ketoconazole and climbazole regarding mean growth rate (MGR) of frond number, characterizing them as highly toxic. Toxicity in terms of corrected EC_{50} (YFN) was highest for climbazole and by factors of 4, 22 and 86 lower for ketoconazole, BDDA and fluconazole, respectively (Figure 1). As EC_x for MGR of frond number had to be extrapolated

beyond tested BDDA concentrations and curve fits were better for YFN, the latter endpoint was used for further comparisons.

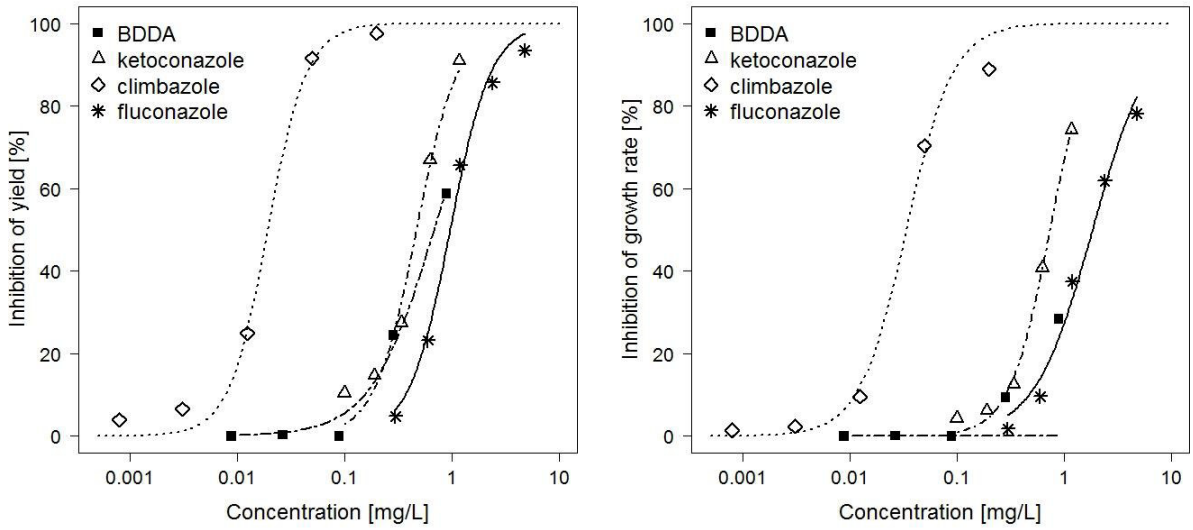


Figure 1: Dose-response curves for inhibition of *L. minor* based on yield (left) and mean growth rate (right) of frond number and nominal concentrations of BDDA (benzyldimethyldodecylammonium, cation), ketoconazole, climbazole and fluconazole.

Toxicity toward water plants such as *Lemna* is not a standard endpoint required for the environmental risk assessment of chemicals regulated under REACH (such as climbazole and BDDA) or as pharmaceuticals (fluconazole and ketoconazole). However, in the case of climbazole and several azole fungicides used in agriculture, *Lemna* proved to be more sensitive than the standard test organisms algae, *Daphnia* or fish (Richter *et al.*, 2013). This was confirmed in the present study for azoles used as antimycotics as the corrected EC_{50} (YFN) of ketoconazole in *L. minor* was about factor 40 lower than the acute EC_{50} of 1.8 mg/L in *D. magna* (Haeba *et al.*, 2008) and the *L. minor* EC_{50} of fluconazole was about factor 4 and 87, respectively, lower than the effect concentrations for *Pseudokirchneriella subcapitata* (EC_{50} of 4.59 mg/L, Chen *et al.*, 2014) and *D. magna* (acute EC_{50} >100 mg/L, Kim *et al.*, 2009). In contrast, *Lemna* appeared to be slightly less sensitive to BDDA than algae and crustaceans based on reported acute EC_{50} of 0.016 mg/L for *D. magna* (Chen *et al.* 2014), and of 0.04 mg/L for *P. subcapitata* and also *D. magna* (referring to measured concentrations, Uhl *et al.*, 2005).

Terrestrial phytotoxicity:

All bioassays with *B. napus* met the respective validity criteria of the test guideline (see supplements Table S4). All test substances inhibited the growth of *B. napus* in a concentration-dependent manner (Figure 2), allowing the estimation of effective concentrations (EC_{10}, EC_{50}) and NOECs for BDDA, ketoconazole, climbazole and fluconazole (Table 4, toxicity estimates based on shoot length and EC_{20} are provided in Table S6).

Seedling emergence was affected only by BDDA, climbazole and fluconazole. Survival of seedlings was not inhibited by the substances except for at the highest test concentration of fluconazole. Shoot growth was generally more affected than seedling emergence by all test substances, with biomass mostly being more sensitive than shoot length. Phytotoxicity of climbazole and fluconazole was very similar (based on EC_{50} for biomass) and by factor 22 and 57 times greater than that of BDDA and ketoconazole, respectively (Figure 2). Yet, when considering the dissipation of BDDA and ketoconazole in the aquatic biotests (Table 2) and the reports on their degradability during WWT (Zhang *et al.*, 2011, Peng *et al.*, 2012), it cannot be excluded that their comparatively low terrestrial toxicity was at least partially due to degradation of the two substances during the test. In other words, correction for actual soil concentrations probably would result in toxicity estimates more similar to those of fluconazole and climbazole. Indeed, measured soil concentrations of BDDA (Figure 3) indicate substantial deviation between nominal BDDA concentration and those determined at test end, which could be explained by degradation of BDDA in soil.

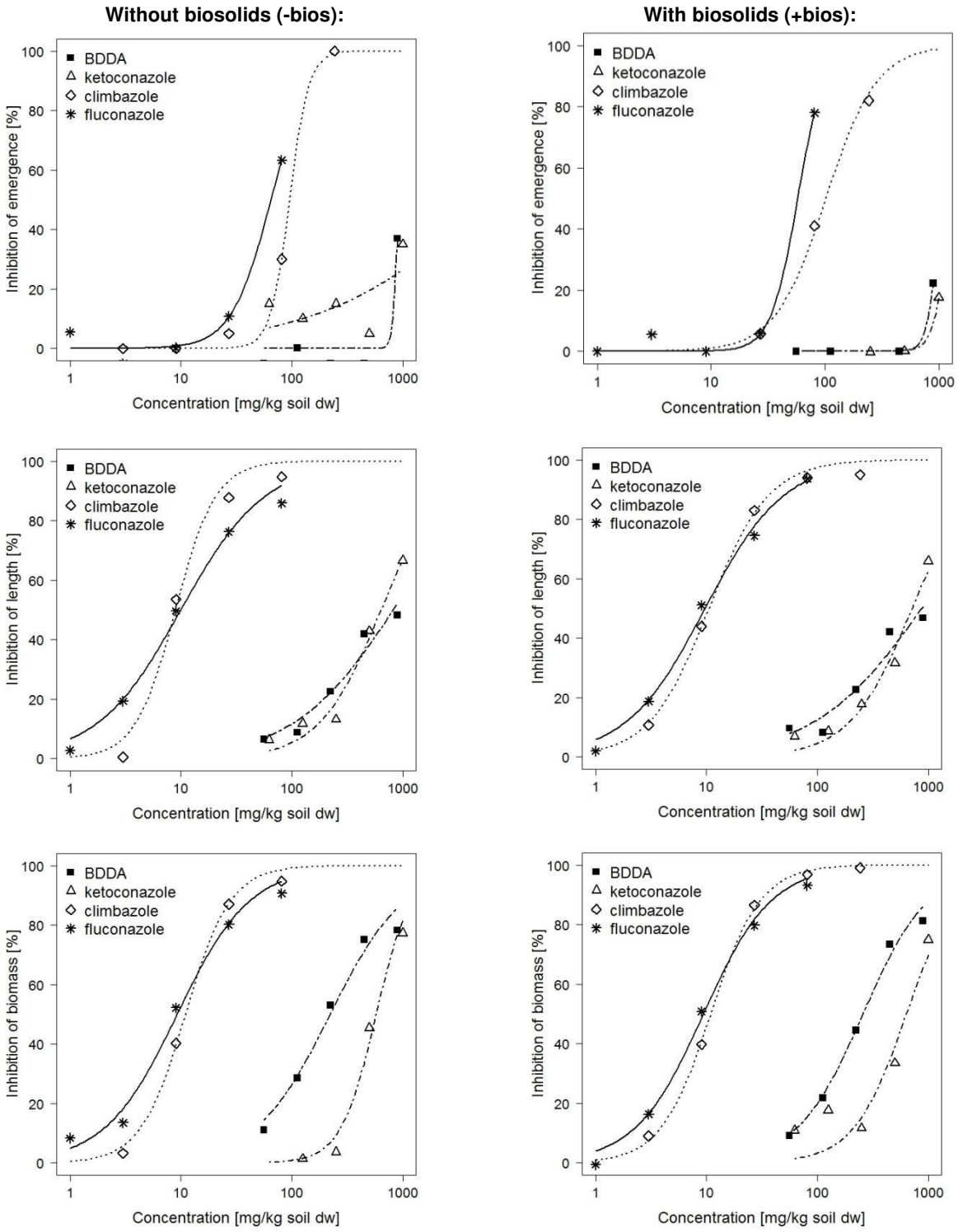


Figure 2: Concentration-response curves for inhibition of *B. napus* emergence rate (top), shoot length (middle) and shoot biomass (bottom) with increasing nominal soil concentrations of BDDA (benzyldimethyldodecylammonium, cation), ketoconazole, climbazole and fluconazole. Left: without, right: with biosolids amendment (3.8 g biosolids d.w./kg soil d.w.).

Comparable to the case of aquatic phytotoxicity, terrestrial phytotoxicity of the antimycotics climbazole and fluconazole was in the same range as that of agricultural fungicides, e.g. that of tebuconazole (EC_{50} of 4 mg/kg soil for shoot length, EC, 2007).

Table 4: Effective concentrations (EC_x with 95% confidence intervals, CI) and no observed effect concentrations (NOECs) in mg/kg d.w. for emergence and biomass (fresh weight) of *B. napus* of test in soil (- bios) and soil amended with biosolids at 3.8 g d.w./kg soil d.w. (+ bios).

	BDDA		Ketoconazole		Climbazole		Fluconazole	
EC_x (95% CI)	(-bios)	(+bios)	(-bios)	(+bios)	(-bios)	(+bios)	(-bios)	(+bios)
Emergence								
NOEC	448	≥896	≥1000	≥1000	27	81	27	27
EC_{10}	817	798	>1000	>1000	49.9	76.8	22.3	19.9
	(n.d.)	(n.d.)	(n.d.)	(n.d.)	(7.1-92.7)	(n.d.)	(0-50.5)	(0-43.4)
EC_{50}	923*	1045*	>1000	>1000	94.1	92.5	67.7	55.3
	(444-1402)	(n.d.)	(n.d.)	(n.d.)	(43.4-145)	(n.d.)	(8.81-127)	(13.1-97.5)
Biomass								
NOEC	<56	224	500	250	3	3	<1.0	3
EC_{10}	40.2	57.5	257	192	4.3	3.52	1.77	1.99
	(23.7-56.6)	(39.0-76.1)	(145-369)	(62.2-322)	(3.61-4.99)	(2.70-4.34)	(1.18-2.36)	(1.08-2.90)
EC_{50}	222	258	578	636	10.2	10.9	9.24	9.38
	(185-259)	(223-293)	(440-715)	(508-764)	(9.37-11.0)	(9.79-12.0)	(7.90-10.6)	(7.34-11.4)

* Indicates that value was derived from extrapolation. BDDA = benzyldimethyldodecylammonium (cation).

Influence of sludge amendment on terrestrial phytotoxicity:

Growth of *B. napus* in the controls was significantly enhanced (by 19%) in the +bios treatment compared to the -bios treatment based on shoot length ($p<0.001$). Hence, no phytotoxic effects were observed related to biosolids applied at usual agricultural rates.

While EC_{50} values for growth inhibition by ketoconazole and BDDA tended to be higher in the +bios compared to in the -bios treatment, no significant influence of the biosolids amendment on the parameters of the concentration-response curves (i.e., EC_{50} and slope *b*) was detected for any of the test substances. The only exception was a significantly ($p<0.05$) flatter curve in the case of climbazole +bios (Table S7); a single finding that might simply be related to the sheer number of comparisons. Thus, the hypothesis that sludge amendment would reduce the effects of sorptive substances as reported in previous studies (Erikkson *et al.*, 2008, Wu *et al.*, 2009, Peña *et al.*, 2014) was not supported by the present results.

The non-significant impact of biosolids amendment in the present study was probably due to the comparatively low biosolids application rate of 5 t/ha corresponding to 3.8 g d.w./kg soil d.w. This increased the soil C_{org} (1.0%, LUFA 2.3) by approximately 12%, to a final content of 1.1%. In

contrast, most studies where effects from biosolids or sludge amendment were detected used substantially higher sludge application rates. For example, soil amendment with approximately 20 g/kg d.w. sewage sludge resulting in a soil C_{org} of approximately 1.8% significantly reduced the uptake into ryegrass and phytotoxicity of pesticides (Peña *et al.*, 2014). Further, sewage sludge amendment at 12-29 t/ha significantly reduced triclosan uptake into plant roots, which was likewise attributed to the increased C_{org} of the soil (Prosser *et al.*, 2014). It can be assumed that with increasing contact time between the sludge-soil matrix and pollutants (e.g. by pre-incubation of the substance with sludge before adding to soil), sequestration processes may further reduce their bioavailability and toxicity (Reid *et al.*, 2000).

Overall, our results provide evidence that biosolids or sewage sludge applied at a realistic field application rate of 5 t/ha do not detectably influence the phytotoxicity of co-applied organic micropollutants. Hence, it can be concluded that standard biotests conducted without sludge amendment are sufficiently conservative without being over-protective to assess the effects of sludge-born organic micropollutants on terrestrial plants.

Uptake of BDDA into *B. napus*:

At test end, i.e. 19 days after test start, measured BDDA concentrations in soil (Figure 3, Table S8) amounted to less than 32% of nominal test concentrations with no significant influence by the biosolids treatment (p=0.718, one-way ANOVA).

BDDA was found in the shoot tissue of all exposed plants. The measured plant concentration of BDDA increased with the nominal soil concentration, reaching up to 4.3 mg/kg d.w. in *B. napus* shoots (supplements Table S8). Based on the total BDDA mass applied per pot and detected in the respective shoots (using an average soil mass of 0.38 kg d.w. and the sum shoot dry weight per pot, calculations see Table S8), a negligible BDDA fraction of less than 0.001% was taken up by the plant shoots. Thus, the low recovery of soil concentrations at test end was not caused by uptake into plants, but rather provides evidence for substantial degradation of BDDA in soil. However, it can be assumed that sorption onto and uptake into roots of *B. napus*, that were not included in the measurement here, at least partly contributed to the low recovery in soil. Further, metabolisation of BDDA inside the plant may lead to underestimating of uptake as it has been found that in carrot cells the conjugates' concentrations of the antibacterial agent triclosan were up to factor 5 higher than the parent compound itself (Macherius *et al.*, 2012).

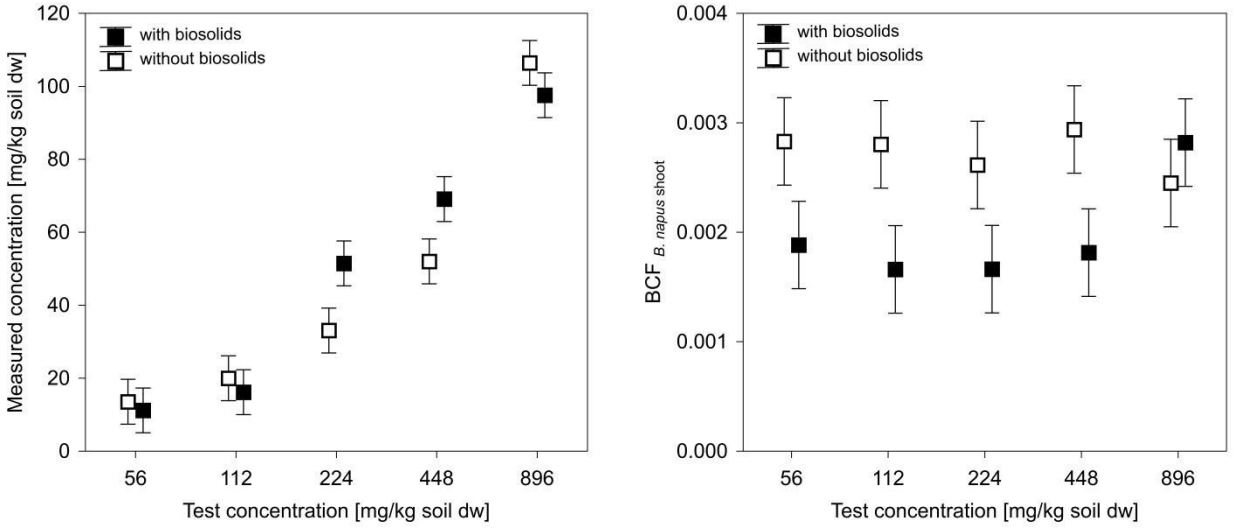


Figure 3: Left: BDDA (benzyldimethyldodecyl ammonium, cation) measured concentration in soil at the end of the plant test (19 days after test start); right: BDDA bioconcentration factor (BCF) from soil (nominal concentrations) to *B. napus* shoot (mean with standard error), n = 4 replicates per treatment.

Average BDDA concentrations in shoot tissue tended to be lower in the +bios treatments, but were not significantly different from those in -bios (one-way ANOVA, p=0.71). Yet, the bioconcentration factor from soil (nominal concentrations) to shoot ($BCF_{B.\ napus\ shoot}$) was found to be significantly reduced by the biosolids amendment (two-way ANOVA, p=0.005, Figure 3), while neither BDDA concentration level nor the interaction between the two factors (concentration level*biosolids treatment) were found to have a significant influence (both p>0.05) on the BCF. The independence from concentration levels indicates that equilibrium was established between BDDA concentrations in soil and plant shoot. Overall, $BCF_{B.\ napus\ shoot}$ of on average 0.0027 (-bios) and 0.0020 (+bios) demonstrated that BDDA concentrations in *B. napus* shoot tissue were at least factor 100 smaller than nominal concentrations in soil. The $BCF_{B.\ napus\ shoot}$ would still be smaller than 0.02, even if based on measured soil concentrations at test end instead of much higher nominal soil concentrations.

For other PPCPs, substantially higher plant BCFs than that for BDDA have been reported (Holling *et al.*, 2012, Tanoue *et al.*, 2012, Prosser *et al.*, 2014). For example, carbamazepine was accumulated approximately 100-fold from soil fortified at 2.6 µg/kg to cabbage shoot and root tissue with mean concentrations of 237 µg/kg and 329 µg/kg, respectively (Holling *et al.*, 2012). This is probably related to the faster uptake of an uncharged molecule such as carbamazepine compared to a charged molecule such as BDDA (Trapp, 2000) and to the better internal transport of substances with medium lipophilicity (log K_{ow} from 1.5 to 3) that can cross hydrophobic membranes (e.g. of root endodermis) and also move into the shoot via the aqueous xylem sap (Trapp, 2000, Tanoue *et al.*,

2012). In accordance with the present findings, the bioconcentration of carbamazepine in cabbage was found to be substantially lower in soil amended with biosolids (approximately factor 5 and 28 for shoot and root, respectively) than in soil fortified with carbamazepine (Holling *et al.*, 2012).

To conclude, sludge amendment reduced the bioconcentration of BDDA in plants, which provides evidence for reduced bioavailability. However, as mentioned above, this reduction did not result in significantly reduced growth inhibition in these plants.

Phytotoxicity and mode of action:

Visual effects observed in *Lemna* exposed to ketoconazole and BDDA were a general reduction of frond size and number and slightly chlorotic fronds. In contrast, plants exposed to climbazole (Richter *et al.*, 2013) and fluconazole exhibited markedly smaller, crisp fronds of darker green color and formed clusters that did not detach from the colonies. This phenomenon was likewise observed in *L. minor* treated with uniconazole P, a plant growth regulator that is known to inhibit gibberellin biosynthesis (Inada *et al.*, 2000). Similarly, specific symptoms such as reduced shoot elongation, in combination with smaller, greener and crisper leaves were observed in *B. napus* affected by ketoconazole, climbazole and fluconazole (Figure 4). Regarding the here tested azoles, only ketoconazole was previously demonstrated to specifically inhibit the biosynthesis of brassinosteroids in plants (Oh *et al.*, 2012).

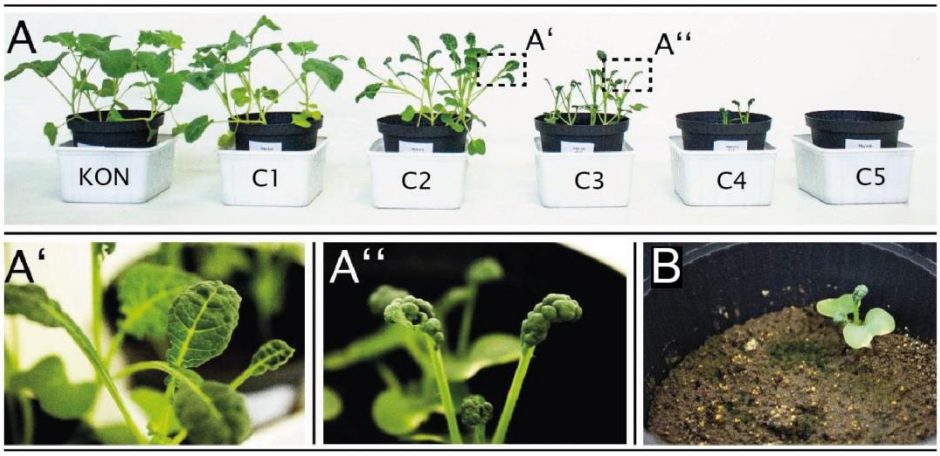


Figure 4: Morphological effects of fluconazole on *B. napus* at test end. A: Treatment concentrations ranging from 1-81 mg/kg soil d.w. A': leaf at 3 mg/kg; A'': leaf at 9 mg/kg; B: plant at 81 mg/kg.

Side effects of azole fungicides to non-target organisms relate to the highly conserved amino acid sequence of CYP51, which is involved in the biosynthesis of sterols throughout all eukaryotic kingdoms (Rademacher, 2000). Bulk sterols constitute ubiquitous components of the plasma

membranes (Lepesheva and Waterman, 2007), while sterols also serve as precursors for mammalian steroid hormones, insect ecdysteroids (Lepesheva and Waterman, 2007) and for certain plant hormones. Gibberellins promote longitudinal growth, germination, and fruit setting (Rademacher, 2000), and brassinosteroids control cell elongation and proliferation, seedling development, and stress tolerance (Oh *et al.*, 2012). To what extend an azole affects the biosynthesis of bulk sterols or that of phytohormones depends on the individual molecular structure being similar either to lanosterol (precursor of sterols) or rather to ent-kaurene (precursor of ent-kaurenoic acid) (Rademacher, 2000). Given the observed morphological alterations in *L. minor* and *B. napus* caused by ketoconazole, climbazole and fluconazole, it can be concluded that all these three antimycotics specifically interfere with phytohormone biosynthesis.

Similar to azoles, certain QACs such as chlormequat chloride (CCC) and dimethylmorpholinium chloride (17-DMC) are known to block the biosynthesis of gibberellins (Rademacher, 2000, Saito *et al.*, 1996), and are therefore applied as plant growth retardants (Miliuviene *et al.*, 2003). However, QACs encompass a variety of structures and thus possess various properties and efficacies depending on their individual side chains (Gerba, 2015). As in the present study, no specific growth retarding symptoms were observed in BDDA-exposed *B. napus* and *L. minor*, a specific phytohormone-influencing mode of action appears unlikely for BDDA. Nevertheless, BDDA toxicity to *L. minor* was pronounced and even higher than that of fluconazole.

For ionisable organic substances such as the tested compounds, their toxicity is not only related to the hydrophobicity of the uncharged molecule but also depends on the pH-specific partitioning. Particularly for weak bases such as the tested azoles, sorption to soil is hard to predict quantitatively as electrostatic attraction is mostly stronger than hydrophobic partitioning to organic matter (Schaffer and Licha, 2015). The same is true for BDDA because as a cationic surfactant it is permanently cationic, i.e. without the dissociated chloride, independent of the pH (Gerba, 2015). In the present study, based on their pK_a (Table 1) and average test pH, only fluconazole was uncharged, while climbazole and ketoconazole existed mostly in the cationic form (approximately 95% and 67%, respectively, in aqueous medium with pH 6.2, and 83% and 34%, respectively, in soil at pH 6.8). Thus, the comparatively low terrestrial phytotoxicity of ketoconazole compared to its high aquatic phytotoxicity is probably related to its strong sorption to soil of both the hydrophobic neutral and the cationic molecules and its thereby reduced bioavailability, as it has been concluded for other PPCPs (Holling *et al.*, 2012). The low hydrophobicity and uncharged character of fluconazole, in contrast, is likely responsible for its comparatively high terrestrial phytotoxicity. Yet, even with the greater portion being cationic, climbazole exerted high aquatic as well as terrestrial toxicity, underlining that ecotoxicity is hard to predict based on physicochemical properties alone. This may at least partly be due to the highly specific mode of action of the here investigated azole antimycotics.

Environmental relevance of observed toxicity:

In a recent monitoring of climbazole in the river Yangtze, climbazole was detected at all 24 sampling locations at concentrations ranging from 0.14-2.12 ng/L in water and from 0.17-4.41 µg/kg in sediments (Zhang *et al.*, 2015). Data on biodegradability indicate that climbazole and fluconazole are rather persistent and can potentially accumulate in soil based on their half-lives of 175 days (Chen *et al.*, 2013) and 73-85 days (Garcia-Valcarcel *et al.*, 2012), respectively, in sludge-amended soil. BDDA was shown to rapidly sorb to biomass, to inhibit sludge respiration at high concentrations (half-saturation inhibition constant of 0.28-0.15 mg/L) and to serve as carbon source in the absence of readily available sugars (Zhang *et al*., 2011). Biodegradability can also be assumed for ketoconazole during WWT (Peng *et al.*, 2012). Yet, once the substances are bound to the soil surface and sequestered into organic matter, their degradation may be hindered (Reid *et al.*, 2000) so that it cannot be excluded that their residues potentially accumulate in sludge-amended soil.

Preliminary hazard quotients (HQs) for chronic phytotoxicity were calculated by dividing measured environmental concentrations by the determined EC_{10} values (Table 5).

A few studies are available that report (often only as minimum-maximum range) measured concentrations for effluent and sewage sludge of BDDA (Clara *et al.*, 2007, Kreuzinger *et al.*, 2007, Martinez-Carballo *et al.*, 2007), ketoconazole (Peng *et al.*, 2012, Casado *et al.*, 2014, Garcia-Valcarcel *et al.*, 2011, Van de Steene *et al.*, 2010, Macikova *et al.*, 2014), climbazole (Wick *et al.*, 2010, Chen *et al.*, 2013, Chen *et al.*, 2014) and fluconazole (Peng *et al.*, 2012, Casado *et al.*, 2014, Macikova *et al.*, 2014, Loos *et al.*, 2013, Garcia-Valcarcel *et al.*, 2011).

Table 5: Preliminary hazard quotients (HQs) of determined effective concentrations for test substances and measured environmental concentrations (MECs) in effluent and sewage sludge obtained from literature.

Substance	EC_{10} *L. minor* YFN [µg/L]	MEC effluent [µg/L]	HQ effluent, geomean (max)	EC_{10} *B. napus* BM [mg/kg d.w.]	MEC sludge [µg/kg d.w.]	HQ sludge, geomean (max)
BDDA	67.0	0.175 (0.03 - 0.50) [1]; 0.16 (unfiltered municipal) [3]; 4.1 (unfiltered diary) [3]	0.007 (0.06)	40	25000 [2]; 73 (6 - 3600, river sediments) [3]	0.03 (0.6)
Ketoconazole	17.7	0.02 (<0.05 - 0.04) [5]; 0.005 (<0.005 - 0.035) [7]; 0.043 (0.006 - 0.077) [8]	0.001 (0.002)	257	535 (62 - 4449) [6]; 335 (126-490) [4]	0.002 (0.02)
Climbazole	5.34	0.378 (0.312 - 0.443) [9]; 0.407 [10]	0.02 (0.08)	4	1160 [9]; 165 [11]	0.1 (0.3)
Fluconazole	371	3.304 (0.182 - 6.426) [8]; 0.14 [4]; 0.04 (0.02 - 0.10) [5]; 0.067 (0.598) [12]	0.0003 (0.009)	2	2 (<2 - 58) [6]	0.001 (0.03)

L. minor: EC_{10} for yield frond number based on overall recovery (BDDA, ketoconazole and climbazole) or nominal concentrations (fluconazole); *B. napus*: EC_{10} for biomass (shoot fresh weight); MECs given as mean concentration with min-max range [1], max [2, 10], median with min-max [3-9], mean [11] or median with max [12] ; (values <LOQ were considered with LOQ). HQ=MEC/EC_{10} (MEC as geometric mean and maximum of all reported MECs). BDDA = benzyldimethyldodecylammonium (cation).

References: [1] Clara et al., 2007; [2] Martınez-Carballo et al., 2007; [3] Kreuzinger et al., 2007; [4] Peng et al., 2012; [5] Casado et al., 2014; [6] Garcia-Valcarcel et al., 2011; [7] Van de Steene et al., 2010; [8] Macikova et al., 2014; [9] Wick et al., 2010; [10] Chen et al., 2014; [11] Chen et al., 2013; [12] Loos et al., 2013.

For the HQ estimation, median concentrations (cited or calculated from literature data) as well as maximum concentrations were used to estimate average and worst-case hazard. The hazard estimation is rather indicative, because the preliminary HQs do not include dilution factors from sludge to soil or from wastewater to surface water, and because no safety factors were applied to effective concentrations. Secondly, available information on measured environmental concentrations is scattered and likely not representative, while exposure pathways such as run-off from sludge-amended fields (Topp *et al.*, 2008) or repeated sludge amendment at elevated rates (Chen *et al.*, 2013) are not taken into account. Maximum HQs for aquatic plants ranged from 0.002 for ketoconazole to 0.08 for climbazole, and for terrestrial plants from 0.02 for ketoconazole to 0.6 for BDDA. While no immediate risks are indicated (i.e. no HQ >1) these estimates demonstrate greatest environmental risk with a safety margin of less than factor 15 for BDDA and climbazole both for the aquatic and the terrestrial compartment.

Conclusions

The present study demonstrates that wastewater-born antimycotics exhibit high phytotoxicity to aquatic and terrestrial plants and at similar concentration ranges as structurally related fungicides used in agriculture. Yet, the high susceptibility of aquatic plants such as water lentils is currently not taken into account in the environmental risk assessment required for ingredients of PPCPs. Based on the observed symptoms it can be concluded that ketoconazole, climbazole and fluconazole specifically interact with the biosynthesis of phytohormones, similar to substances used as plant growth regulators in agriculture. The co-application of sludge at a realistic rate did not significantly reduce the substances' toxicity so that standard ecotoxicity testing appears not to be overprotective for exposure of terrestrial plants via sludge. Based on preliminary HQs, the tested substances appear to pose no immediate risk for plants, particularly in the case of the two pharmaceuticals, ketoconazole and fluconazole. Yet, maximum HQs of >0.1 for BDDA and climbazole in digested sewage sludge and of >0.01 in effluent leave a small margin of safety that should be further evaluated by compiling more data on actual environmental concentrations.

Acknowledgement

This work was supported by the project ROUTES funded by the European Union (Contract No 265156, FP7 2007-2013, THEME [ENV.2010.3.1.1-2], Innovative system solutions for municipal sludge treatment and management) and project ELaN (support code 033L025A) funded by the German Federal Ministry of Education and Research (BMBF). We are grateful to Ed Topp and Lyne Sabourin for the kind supply of the biosolids and to Jörg Oehlmann for valuable discussions.

References

Beecher, N., 2008. Moving forward the sustainable and welcome uses of a global resource, in: LeBlanc, R.J., Matthews, P., Richard R.P. (Eds.), Global Atlas of Excreta, Wastewater Sludge, and Biosolids Management. United Nations Human Settlements Programme (UN-HABITAT), Nairobi, Kenya, pp. 16-83.

Braguglia, C.M., Coors, A., Gallipoli, A., Gianico, A., Guillon, E., Kunkel, U., Mascolo, G., Richter, E., Ternes, T.A., Tomei, M.C., Mininni, G., 2014. Environmental science and pollution, 22, 7216-7235.Casado, J., Rodríguez, I., Ramil, M., Cela, R., 2014. Selective determination of antimycotic drugs in environmental water samples by mixed-mode solid-phase extraction and liquid chromatography quadrupole time-of-flight mass spectrometry. Journal of Chromatography A 1339, 42–49.

Chen, Y., Geurts, M., Sjollema, S.B., Kramer, N.I., Hermens, J.L.M., Droge, S.T.J., 2014. Acute toxicity of the cationic surfactant C12-benzalkonium in different bioassays: How test design affects bioavailability and effect concentrations: Acute toxicity of the cationic surfactant C12-benzalkonium. Environmental Toxicology and Chemistry 33, 606–615.

Chen, Z.-F., Ying, G.-G., Ma, Y.-B., Lai, H.-J., Chen, F., Pan, C.-G., 2013. Typical Azole Biocides in Biosolid-Amended Soils and Plants Following Biosolid Applications. J. Agric. Food Chem. 61, 6198–6206.

Clarke, B.O., Smith, S.R. 2011. Review of 'emerging' organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids. Environ. Int. 37, 226-247.

Cogger, C.G., Forge, T.A., Nielsen, G.H. 2006. Biosolids recycling: Nitrogen management and soil ecology. Can. J. Soil Sci. 86, 613–620

Corrêa, J.C.R., Reichman, C., Salgado, H.R.N., Vianna-Soares, C.D., 2012. Performance characteristics of high performance liquid chromatography, first order derivative UV spectrophotometry and bioassay for fluconazole determination in capsules. Química Nova 35, 530–534.

EC (European Comission), 2014, registration of benzododecinium chloride: http://ec.europa.eu/transparency/ regdoc/rep/3/2014/EN/3-2014-5391-EN-F1-1-ANNEX-1.Pdf.

EC (European Comission) Draft Assessment Report on Tebuconazole, Volume 1, February 2007: http://ec.europa.eu/sanco_pesticides.

EMA/888361/2011 assessment report on diflucan (fluconazole): http://www.ema.europa.eu/docs/en_GB/document_library/Referrals_document/Diflucan_30/WC500121953.pdf, European Medicines Agency, London, UK, 2012.

EMA/CHMP/534845/2014 assessment report on ketoconazole: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/003906/WC500181493.pdf, European Medicines Agency, London, UK, 2014.

Eriksson, E., Christensen, N., Ejbye Schmidt, J., Ledin, A., 2008. Potential priority pollutants in sewage sludge. Desalination, 10th IWA International Specialized Conference on Diffuse Pollution and Sustainable Basin Management 18–22 September 2006, Istanbul, Turkey 10th IWA International Specialized Conference on Diffuse Pollution and Sustainable Basin Management 226, 371–388.

Ferk, F., Misik, M., Hoelzl, C., Uhl, M., Fuerhacker, M., Grillitsch, B., Parzefall, W., Nersesyan, A., Micieta, K., Grummt, T., Ehrlich, V., Knasmuller, S., 2007. Benzalkonium chloride (BAC) and dimethyldioctadecyl-ammonium bromide (DDAB), two common quaternary ammonium compounds, cause genotoxic effects in mammalian and plant cells at environmentally relevant concentrations. Mutagenesis 22, 363–370.

Gerba, C.P., 2015. Quaternary Ammonium Biocides: Efficacy in Application. Applied and Environmental Microbiology 81, 464–469.

Haeba, M., Hilscherová, K., Mazurová, E., Bláha, L., 2008. Selected endocrine disrupting compounds (Vinclozolin, Flutamide, Ketoconazole and Dicofol): Effects on survival, occurrence of males, growth, molting and reproduction of Daphnia magna. Environmental Science and Pollution Research 15, 222–227.

Holling, C.S., Bailey, J.L., Vanden Heuvel, B., Kinney, C.A., 2012. Uptake of human pharmaceuticals and personal care products by cabbage (*Brassica campestris*) from fortified and biosolids-amended soils. Journal of Environmental Monitoring 14, 3029.

Inada, S., Tominaga, M., Shimmen, T., 2000. Regulation of root growth by gibberellin in Lemna minor. Plant Cell Physiol. 41, 657–665.

Kim, J.-W., Ishibashi, H., Yamauchi, R., Ichikawa, N., Takao, Y., Hirano, M., Koga, M., Arizono, K., 2009. Acute toxicity of pharmaceutical and personal care products on freshwater crustacean (Thamnocephalus platyurus) and fish (Oryzias latipes). J Toxicol Sci 34, 227–232.

Klärschlammverordnung (AbfKlärV) vom 15. April 1992 (BGBl. I S. 912), zuletzt geändert durch Artikel 5 Absatz 12 des Gesetzes vom 24. Februar 2012 (BGBl. I S. 212).

Langdon, K.A., Warne, M.S.J., Kookana R.S., 2010. Aquatic hazard assessment for pharmaceuticals, personal care products and endocrine-disrupting compounds form biosolids-amended land. Integr Environ Assess Manag 6, 663–676.

Lepesheva, G.I., Waterman, M.R., 2007. Sterol 14α-demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms. Biochimica et Biophysica Acta (BBA) - General Subjects 1770, 467–477.

Macherius A., Eggen T., Lorenz W., Moeder M., Ondruschka J., Reemtsma T., 2012, Metabolization of the bacteriostatic agent triclosan in edible plants and its consequences for plant uptake assessment. Environmental Science and Technology 46, 10797-10804.

Martínez-Carballo, E., González-Barreiro, C., Sitka, A., Kreuzinger, N., Scharf, S., Gans, O., 2007. Determination of selected quaternary ammonium compounds by liquid chromatography with mass spectrometry. Part II. Application to sediment and sludge samples in Austria. Environmental Pollution 146, 543–547.

Miliuviene, L., Novickiene, L., Jurevicius, J., 2007. Oilseed rape growth regulation by compounds 3-DEC and 17-DMC. Botanica Lithuanica 13, 115–121.

Oh, K., Yamada, K., Asami, T., Yoshizawa, Y., 2012. Synthesis of novel brassinosteroid biosynthesis inhibitors based on the ketoconazole scaffold. Bioorganic & Medicinal Chemistry Letters 22, 1625–1628.

OECD. 2006a. OECD guideline 221: *Lemna sp.* growth inhibition test. Organisation for Economic Co-operation and Development, Paris, France.

OECD. 2006b. OECD guideline 208: Terrestrial plant test: Seedling emergence and seedling growth test. Organisation for Economic Co-operation and Development, Paris, France.

Peña, A., Mingorance, M.D., Guzmán, I., Sánchez, L., Fernández-Espinosa, A.J., Valdés, B., Rossini-Oliva, S., 2014. Protecting effect of recycled urban wastes (sewage sludge and wastewater) on ryegrass against the toxicity of pesticides at high concentrations. Journal of Environmental Management 142, 23–29.

Peng, X., Huang, Q., Zhang, K., Yu, Y., Wang, Z., Wang, C., 2012. Distribution, behavior and fate of azole antifungals during mechanical, biological, and chemical treatments in sewage treatment plants in China. Sci.Total Environ. 426, 311–317.

Prosser, R.S., Lissemore, L., Topp, E., Sibley, P.K., 2014. Bioaccumulation of triclosan and triclocarban in plants grown in soils amended with municipal dewatered biosolids: TCS and TCC bioaccumulation. Environmental Toxicology and Chemistry 33, 975–984.

Rademacher, W., 2000. Growth retardants: Effects on gibberellin biosynthesis and other metabolic pathways. Annual Review of Plant Physiology and Plant Molecular Biology 51, 501–531.

Reid, B., 2000. Bioavailability of persistent organic pollutants in soils and sediments—a perspective on mechanisms, consequences and assessment. Environmental Pollution 108, 103–112.

Richter, E., Wick, A., Ternes, T.A., and Coors, A. (2013). Ecotoxicity of climbazole, a fungicide contained in antidandruff shampoo. Environmental Toxicology and Chemistry 32, 2816–2825.

Richter, E., Roller, E., A., Kunkel, U., Ternes, T.A., and Coors, A. (2015). Assessing the ecological long-term impact of wastewater irrigation on soil and water based on bioassays and chemical analyses. Water Research, accepted.

Ritz, C., Streibig, J.C., 2005. Bioassay analysis using R. Journal of Statistical Software 12, 1–22.

Saito, T., Yamane, H., Sakurai, A., Murofushi, N., Takahashi, N., Kamiya, Y., 1996. Inhibition of ent-Kaurene Synthase by Quaternary Ammonium Growth Retardants. Bioscience, Biotechnology, and Biochemistry 60, 1040–1042.

Schaffer, M., Licha, T., 2015. A framework for assessing the retardation of organic molecules in groundwater: Implications of the species distribution for the sorption-influenced transport. Science of The Total Environment 524-525, 187–194.

Schowanek, D., Carr, R., David, H., Douben, H., Hall, J., Kirchmann, H., Patria, L., Sequi, P., Smith, S., Webb, S., 2004. A risk-based methodology for deriving quality standards for organic contaminants in sewage sludge for use in agriculture-Conceptual Framework. Regulatory Toxicology and Pharmacology 40, 227–251.

Skiba, M., Skiba-Lahiani, M., Marchais, H., Duclos, R., Arnaud, P., 2000. Stability assessment of ketoconazole in aqueous formulations. Int J Pharm 198, 1–6.Tanoue, R., Sato, Y., Motoyama, M., Nakagawa, S., Shinohara, R., Nomiyama, K., 2012. Plant Uptake of Pharmaceutical Chemicals Detected in Recycled Organic Manure and Reclaimed Wastewater. J. Agric. Food Chem. 60, 10203–10211.

Topp, E., Monteiro, S.C., Beck, A., Coelho, B.B., Boxall, A.B.A., Duenk, P.W., Kleywegt, S., Lapen, D.R., Payne, M., Sabourin, L., Li, H., Metcalfe, C.D., 2008. Runoff of pharmaceuticals and personal care products following application of biosolids to an agricultural field. Sci.Total Environ. 396, 52–59.

Trapp, S., 2000. Modelling uptake into roots and subsequent translocation of neutral and ionisable organic compounds. Pest Management Science 56, 767–778.

Uhl, M., Gans, O., Grillitsch, B., Fürhacker, M., Kreuzinger, N. Grundlagen zur Risikoabschätzung für quaternäre Ammoniumverbindungen. Umweltbundesamt Wien, 3-85457-774-5 (2005).

Wheeler M.W., Park R.M., Bailer A.J. (2006) Comparing median lethal concentration values using confidence interval overlap or ratio tests, Environmental Toxicology & Chemistry 25 (5), 1441–1444.

Wick, A., Fink, G., Ternes, T.A., 2010. Comparison of electrospray ionization and atmospheric pressure chemical ionization for multi-residue analysis of biocides, UV-filters and benzothiazoles in aqueous matrices and activated sludge by liquid chromatography-tandem mass spectrometry. Journal of Chromatography A 1217, 2088–2103.

Wu, C., Spongberg, A.L., Witter, J.D., 2009. Adsorption and Degradation of Triclosan and Triclocarban in Solis and Biosolids-Amended Soils. J. Agric. Food Chem. 57, 4900–4905.

Zhang, C., Tezel, U., Li, K., Liu, D., Ren, R., Du, J., Pavlostathis, S.G., 2011. Evaluation and modeling of benzalkonium chloride inhibition and biodegradation in activated sludge. Water Research 45, 1238–1246.

Zhang, Q.-Q., Ying, G.-G., Chen, Z.-F., Liu, Y.-S., Liu, W.-R., Zhao, J.-L., 2015. Multimedia fate modeling and risk assessment of a commonly used azole fungicide climbazole at the river basin scale in China. Science of The Total Environment 520, 39–48.

Supplements to "*Phytotoxicity of wastewater-born micropollutants – characterisation of three antimycotics and a cationic surfactant*"

Table S1: pH determined in the biotests with *L. minor*.

	BDDA, initial	BDDA, aged			Ketoconazole		Fluconazole	
pH	d0, d2, d5	d2	d5	d7	d0	d7	d0	d7
Medium	5.5	5.9	6.3	6.5	5.7	7.0	5.5	6.8
C1	5.5	5.9	6.3	6.5	5.7	7.0	5.5	6.9
C2	5.5	5.9	6.3	6.5	5.7	7.2	5.5	6.6
C3	5.5	5.9	6.4	6.0	5.7	6.9	5.5	6.9
C4	5.5	5.8	6.0	6.1	5.7	6.4	5.5	6.4
C5	5.5	5.7	5.7	5.8	5.7	6.6	5.5	6.3
mean (C1 – C5) ± SD	5.5 ± 0.0	6.1 ± 0.3			5.7 ± 0.0	6.8 ± 0.3	5.5 ± 0.0	6.6 ± 0.3

Mean pH of initial (day 0 or day 0, 2 and 5 for BDDA) and aged (day 7 or day 2, 5 and 7 for BDDA) calculated over test solutions (C1–C5). SD = standard deviation. Medium = modified Steinberg medium. BDDA = benzyldimethyldodecylammonium (cation.)

Table S2: Nominal test concentrations applied in the biotests with *L. minor* and *B. napus*.

L. minor [mg/L]				*B. napus* [mg/kg soil dry weight]				
BDDA salt	BDDA+	Ketoco-nazole	Fluco-nazole	BDDA salt	BDDA+	Ketoco-nazole	Climba-zole	Fluco-nazole
CON	CON	CON	CON	CON - bios	CON - bios	CON - bios	CON - bios	CON - bios
-	-	SC	-	CON + bios	CON + bios	CON + bios	CON + bios	CON + bios
0.01	0.00896	0.100	0.300	62.5	56	62.5	3	1
0.03	0.0269	0.185	0.600	125	112	125	9	3
0.10	0.0896	0.342	1.200	250	224	250	27	9
0.32	0.269	0.633	2.400	500	448	500	81	27
1.00	0.896	1.171	4.800	1000	896	1000	243	81

CON = control (modified Steinberg medium); SC = solvent control; CON - bios = LUFA Sp2.3; CON + bio = LUFA Sp2.3 with biosolids amendment of 3.8 g/kg soil.

Analytical methods:

Chemicals

BDDA was analysed via determining the cation of the standard benzyldimethyldodecylammonium bromide (given concentrations refer to the cation if not indicated otherwise). Benzyldimethyldodecylammonium bromide and ketoconazole were obtained from Sigma Aldrich (Seelze, Germany), fluconazole, fluconazole-D_4, ketoconazole-D_8, and benzyldimethyldodecylammonium bromide-D_5 (BDDA-D_5) from Toronto Research Chemicals (North York, Ontario, Canada). Methanol (MeOH) and acetonitrile (ACN, both HPLC-grade) were obtained from Merck (Darmstadt, Germany), ultra-pure water was provided by a Milli-Q system (Merck).

Liquid sample preparation

For ketoconazole, 10 µL of the surrogate standard ketoconazole-D_8 (10 µg/mL) were added to an aliquot of 1 mL of each sample and transferred to an HPLC vial. For fluconazole, samples were diluted with MeOH. The dilution factor was adjusted to obtain a final concentration of fluconazole of 300 µg/L. An aliquot of 1 mL of each diluted samples was transferred to an HPLC vial and 10 µL of the surrogate standard fluconazole-D_4 (10 µg/mL) were added. For BDDA, aliquots of samples with nominal BDDA concentrations of 100 and 1000 µg/L were diluted with MeOH to obtain a final concentration of 10 µg/L. Aliquots of each (diluted) sample of 1 mL were transferred to HPLC vials and 25 µL of the surrogate standard BDDA-D_5 (1 µg/mL) were added.

Plant and soil extraction of BDDA

Prior to the analyses of BBDA in *B. napus*, the shoot samples were ground using a cryo mill. Sample extraction was accomplished by pressurized liquid extraction (PLE) using an Accelerated Solvent Extraction system (ASE 350, Dionex, Idstein, Germany). Thereto, aliquots of 0.1 g of plant material (or total plant material if the mass was <0.1 g) was filled into extraction cells (size = 10 mL) and 750 ng of the isotopic labelled internal standard (BDDA-D_5) were added. Cells were filled with quartz sand and well mixed with the plant material. PLE was conducted in four extraction cycles with MeOH as solvent at a pressure of 100 bar and a temperature of 80 C. The plants extracts were then diluted with MeOH to a final volume of 30 mL and approximately 1 mL thereof was transferred into a HPLC vial. Soil extraction was done analogously to plant extraction, with 0.1-1.0 g of soil used for PLE (depending on the nominal concentration of the soil; 0.1 g for 10 mg/kg and 1.0 g for 63 mg/kg).

HPLC-MS/MS methods

All liquid samples as well as plant extracts were analysed for target substances using an HLPC-MS/MS system consisting of a binary LC pump (1260, Agilent, Waldbronn, Germany) and an API 4000 tandem mass spectrometer (Sciex, Darmstadt, Germany).). The MS was operated in positive ionization mode using electrospray ionization (ESI). Separation of fluconazole and ketoconazole was achieved with a binary gradient of ultra-pure water (mobile phase A1) und acetonitrile (mobile phase B1; ACN, both 0.1% formic acid (FA)) on a Luna C18(2) column, (150 x 2 mm, 3 µm, Phenomenex, Aschaffenburg, Germany). The HPLC program was as follows: flow rate: 350 µL/min; 0 min: 95% A1, 3 min: 95% A1, 9 min: 1% A1, 14 min: 1% A1, 14.1 min: 95% A1, 19 min: 95% A1. BDDA was analyzed using binary gradient of water (mobile phase A2, 10 mM ammonium formate (NH_4FA)) + 0.1% FA) and MeOH (mobile phase B2) on a Hydro-RP column (150 x 3 mm, 4 µm, Phenomenex. The HPLC program was as follows: flow rate: 400 µL/min; 0 min: 90% A2, 1.5 min: 90% A2; 5 min: 25% A2; 12 min: 1% A2; 19 min: 1% A2, 19.1 min: 90% A2, 24.5 min: 95% A2. The column temperature was always 40 °C and the injection volume was always 10 µL. Quantification of analytes was done using the multi reaction monitoring (MRM) mode. For each analyte and internal standard two transitions were monitored (Table S3).

Table S3. Overview of the mass transitions of analytes and internal standards and their optimized mass spectrometric parameters.

Substance	MRM 1 [m/z]	MRM 2 [m/z]	DP [V] (MRM1/MRM2)	CE [eV] (MRM1/MRM2)	CXP [V] (MRM1/MRM2)
BDDA (cation)	304.3/91.1	304.3/212.2	100/100	36/29	12/12
BDDA-D_5	309.3/96.1	309.3/212.2	100/100	40/29	12/12
Fluconazole	307.2/220.3	307.2/238.3	70/70	25/20	15/20
Fluconazol-D_4	311.2/223.2	311.2/242.3	70/70	25/23	17/15
Ketoconazole	531.1/497.1	533.1/491.1	110/125	47/46	10/10
Ketoconazole-D_8	539.1/497.1	539.1/244.1	75/75	43/45	12/12

The limit of quantification (LOQ) of the applied analytical procedures was for aqueous samples 0.50 µg/L (BDDA cation), 5 µg/L (ketoconazole) and 10 µg/L (fluconazole), and 5-50 µg/kg d.w. for BDDA cation and plant tissue (depending on the extracted mass of soil and plant tissue).

Table S4 Measured test concentrations and resulting recovery in biotests with *L. minor*.

Substance	Nominal concen-tration [µg/L]	Measured initial concentration [µg/L]			Measured aged concentration [µg/L]			Recovery as measured initial concentration in fresh solution related to nominal concentration [%]				Recovery as measured concentration in aged solution related to measured initial concentration [%]				Geometric mean of measured concentrations in fresh and aged solutions [µg/L]			Mean recovery of geometric mean measured to nominal concentration [%]	Over all recovery [%]
Day		0	2	5	2	5	7	0	2	5	mean	2	5	7	mean	2	5	7		
BDDA	8.96	1.80	1.50	0.89	0.88	1.50	1.80	20.1	16.8	9.94		48.9	100	202		1.26	1.50	1.27	15.0	
	89.6	100	40.0	77.0	33.0	9.50	3.20	112	44.7	86.0		33.0	23.8	4.16		57.4	19.5	15.7	34.5	
	896	1100	850	960	710	430	500	123	94.9	107	68.2	64.5	50.6	52.1	64.4	884	605	693	81.2	43.6
Ketoconazole	100	31.0					5.0	31.0						16.2				12.4	12.4	
	190	51.1					5.0	26.9						9.78				16.0	8.41	
	340	67.3					5.0	19.8						7.43				18.3	5.39	
	630	339					5.0	53.9						1.47				41.2	6.54	
	1170	1093					86.0	93.4			45.0			7.78	8.54			307	26.2	11.8
Fluconazole	300	n.a					n.a	n.a						n.a				n.a	n.a	
	600	813					703	135						*86.5*				756	126	
	1200	1306					1676	109						128				1479	123	
	2400	2838					2618	118						92.3				2726	114	
	4800	5801					5961	121			121			103	102			5881	123	121

If the mean recovery of measured aged to measured initial concentrations were not within 100% +/- 20% of initial solutions, overall recovery was calculated as geometric mean of the recoveries for each test concentration level and the overall recovery as arithmetic mean of these geometric means. For ketoconazole: values < LOQ in aged test solution were replaced by LOQ. LOQ: 0.50 µg/L for BDDA, 5 µg/L for ketoconazole, 10 µg/L for fluconazole; n.a.: not available due to missing sample. BDDA = Benzyldimethyldodecylammonium (cation).

Table S5: Compliance of the controls of the bioassays with the validity criteria of the respective test guidelines.

Test organism	Test guideline	Validity criteria for the controls	Observed in test
B. napus	OECD 208, 2006	≥ 70% emergence in each pot	BDDA, fluconazole: 95%(- bios), 90% (+ bios) climbazole, ketoconazole: 100% (- bios), 85% (+ bios)
		≥ 90% mean survival of emerged plants	BDDA, fluconazole: 100% (- bios), 100% (+ bios) climbazole, ketoconazole: 100% (- bios), 100% (+ bios)
L. minor	OECD 221, 2002	frond number doubling time < 2.5 days	1.7 (BDDA) 2.0 (fluconazole) 2.0 (ketoconazole)

Table S6: ECx and NOEC values of BDDA (cation), ketoconazole, climbazole and fluconazole determined for *L. minor* based on nominal concentrations and corrected for overall recovery.

	Ecx (95% CI)	BDDA	Ketoconazole	Climbazole	Fluconazole
Yield FN	NOEC	0.0896	0.100	0.0031	0.3
	EC_{10}	0.154	0.178	0.0078	0.371
		(0.112 - 0.196)	(0.115 - 0.241)	(0.0062 - 0.0094)	(0.301 - 0.442)
	EC_{20}	0.267	0.255	0.0109	0.529
		(0.217 - 0.318)	(0.191 - 0.320)	(0.0093 - 0.0126)	(0.458 - 0.600)
	EC_{50}	0.686	0.472	0.0195	0.969
		(0.597 - 0.775)	(0.406 - 0.539)	(0.0171 - 0.0219)	(0.886 - 1.05)
	Parameter b	-1.47	-2.25	-2.39	-2.29
MGR FN	NOEC	0.269	0.190	0.0031	0.3
	EC_{10}	7.09*	0.286	0.0113	0.473
		(0.00 - 28.3)	(0.227 - 0.345)	(0.0079 - 0.0147)	(0.369 - 0.577)
	EC_{20}	7.14*	0.407	0.0170	0.783
		(0.00 - 28.5)	(0.349 - 0.465)	(0.0133 - 0.0207)	(0.662 - 0.904)
	EC_{50}	7.22*	0.743	0.0341	1.85
		(0.00 - 28.8)	(0.678 - 0.808)	(0.0341 - 0.0382)	(1.66 - 2.04)
	Parameter b	-122	-2.3	-1.99	-1.61
Yield BM	NOEC	0.0896	0.100	0.0008	< 0.3
	EC_{10}	0.125	0.133	0.0016	0.096
		(0.0499 - 0.199)	(0.097 - 0.169)	(0.0002 - 0.0029)	(0.00 - 0.192)
	EC_{20}	0.217	0.284	0.003	0.239
		(0.129 - 0.306)	(0.238 - 0.331)	(0.0011 - 0.0049)	(0.077 - 0.400)
	EC_{50}	0.56	1.04	0.0091	1.14
		(0.427 - 0.694)	(0.896 - 1.19)	(0.0056 - 0.0125)	(0.745 - 1.53)
	Parameter b	-1.46	-1.07	-1.25	-0.89
MGR BM	NOEC	0.0896	0.100	0.0031	0.3
	EC_{10}	0.308	0.392	0.0025	0.195
		(0.223 - 0.392)	(0.339 - 0.446)	(0.0002 - 0.0048)	(0.009 - 0.381)
	EC_{20}	0.572	0.872	0.0044	0.765
		(0.492 - 0.652)	(0.798 - 0.947)	(0.0015 - 0.0073)	(0.379 - 1.15)
	EC_{50}	1.65*	3.42*	0.0114	7.92*
		(1.25 - 2.06)	(2.60 - 4.24)	(0.0074 - 0.0155)	(2.59 - 13.3)
	Parameter b	-1.31	-1.02	-1.46	-0.59

Table S6 (continued)

Ecx (95% CI)	BDDA	Ketoconazole	Climbazole	Fluconazole
Overall recovery [%]	43.6	11.8	71	121
Yield FN recovery corrected				
EC_{10}	0.0670	0.0210	0.0055	0.449
EC_{20}	0.1164	0.0301	0.0077	0.640
EC_{50}	0.2992	0.0557	0.0138	1.17
MGR FN recovery corrected				
EC_{10}	3.09	0.0337	0.0080	0.572
EC_{20}	3.11	0.0480	0.0121	0.947
EC_{50}	3.15	0.0877	0.0242	2.24
Yield BM recovery corrected				
EC_{10}	0.0545	0.0157	0.0011	0.116
EC_{20}	0.095	0.034	0.002	0.289
EC_{50}	0.244	0.123	0.006	1.38
MGR BM recovery corrected				
EC_{10}	0.13	0.05	0.00	0.24
EC_{20}	0.25	0.10	0.00	0.93
EC_{50}	0.72	0.40	0.01	9.58

NOEC: No observed effect concentration; ECx: concentration estimate with x% effect; CI: confidence interval; FN = frond number; MGR = mean growth rate; BM = biomass (dry weight). *extrapolated beyond tested concentrations; Re-calculated based on proportional responses from Richter et al. 2013.

Table S7: EC_x and NOEC of BDDA (cation), ketoconazole, climbazole and fluconazole determined for *B. napus* based on nominal concentrations in soil.

	BDDA		Ketoconazole		Climbazole		Fluconazole	
EC_x (95% CI)	(-bios)	(+bios)	(-bios)	(+bios)	(-bios)	(+bios)	(-bios)	(+bios)
Emergence								
NOEC	448	≥ 896	≥ 1000	≥ 1000	27	81	27	27
EC_{10}	817	798	> 1000	> 1000	49.9	76.8	22.3	19.9
	(n.d.)	(n.d.)	(n.d.)	(n.d.)	(7.1 – 92.7)	(n.d.)	(0 – 50.5)	(0 – 43.4)
EC_{20}	854	881	> 1000	9030	63.1	82.3	33.6	29
	(144 - 1565)	(618 - 1145)	(n.d.)	(n.d.)	(21.2 - 104)	(n.d.)	(2.10 - 65.1)	(2.86 - 55.1)
EC_{50}	923*	1045*	> 1000	> 1000	94.1	92.5	67.7	55.3
	(444 - 1402)	(n.d.)	(n.d.)	(n.d.)	(43.4 – 145)	(n.d.)	(8.81 – 127)	(13.1 – 97.5)
Parameter b	-54	-26.9	(n.d.)	-6.13	-3.45	-11.8	-1.98	-2.15
Shoot length								
NOEC	112	< 56	62.5	< 62.5	3	< 3	1	1
EC_{10}	83	75	154	172	3.58	2.71	1.48	1.6
	(37 - 130)	(39.8 - 110)	(96.6 - 212)	(125 - 219)	(1.84 - 5.32)	(2.10 - 3.32)*	(1.06 - 1.89)	(1.18 - 2.03)
EC_{20}	194	185	261	291	5.02	4.47	2.98	3.11
	(129 - 259)	(133 - 237)	(196 - 326)	(239 - 343)	(3.41 - 6.63)	(3.76 -5.19)	(2.39 - 3.58)	(2,52 - 3.71)
EC_{50}	822	873	640	715	8.94	10.5	9.96	9.7
	(565 - 1078)	(638 - 1107)	(541 - 738)	(638 - 792)	(7.20 - 10.7)	(9.50 - 11.6)	(8.67 - 11.3)	(8.49 - 10.9)
Parameter b	-0.96	-0.89	-1.55	-1.54	-2.4	-1.62	-1.15	-1.22
Biomass								
NOEC	< 56	224	500	250	3	3	< 1.0	3
EC_{10}	40.2	57.5	257	192	4.3	3.52	1.77	1.99
	(23.7 - 56.6)	(39.0 - 76.1)	(145 - 369)	(62.2 - 322)	(3.61 - 4.99)	(2.70 - 4.34)	(1.18 - 2.36)	(1.08 - 2.90)
EC_{20}	75.5	100	346	299	5.91	5.34	3.26	3.53
	(54.1 - 96.9)	(77.5 - 123)	(237 - 455)	(165 - 433)	(5.31 - 6.51)	(4.45 - 6.22)	(2.49 - 4.02)	(2.37 - 4.68)
EC_{50}	222	258	578	636	10.2	10.9	9.24	9.38
	(185 - 259)	(223 - 293)	(440 - 715)	(508 - 764)	(9.37 - 11.0)	(9.79 - 12.0)	(7.90 - 10.6)	(7.34 - 11.4)
Parameter b	-1.29	-1.46	-2.71	-1.84	-2.54	-1.94	-1.33	-1.42

NOEC: No observed effect concentration (for emergence and survival applying the Fisher`s Exact Binomial test with Bonferroni Correction, $\alpha = 0.05$, one-sided greater, for shoot length and biomass applying the William t-test, a = 0.05, one-sided smaller, determined on raw data in TOXRAT version 10.2). * Indicates that value was derived from extrapolation. Survival of plants was not affected by the test substances except for fluconazole at the highest test concentration, i.e. with a NOEC > 81 mg/kg d.w. Quotients refer to concentration-response curve parameter (EC_{50} and b) from with biosolids amendment test divided by parameter from without biosolids amendment test and ** indicates significant difference between +bios and –bios.

Table S8: Comparison of *B. napus* concentration-response curves from with and without biosolids amendment by quotients of EC_{50} and parameter *b* (slope).

Substance	Parameter	Quotient	t-value
	emergence, EC_{50}		
BDDA		1.04	0.02
Climbazole		0.98	0.01
Ketoconazole		1.00	NA
Fluconazole		0.82	-0.38
	emergence, *b*		
BDDA		0.50	-0.03
Climbazole		3.42	0.09
Ketoconazole		n.d.	NA
Fluconazole		1.09	0.10
	shoot length, EC_{50}		
BDDA		1.06	0.318
Climbazole		1.17	1.65
Ketoconazole		1.12	1.15
Fluconazole		0.97	-0.226
	shoot length, *b*		
BDDA		0.93	-0.417
Climbazole		0.68	**-2.44***
Ketoconazole		0.99	-0.0184
Fluconazole		1.06	0.442
	biomass, EC_{50}		
BDDA		1.16	1.12
Climbazole		1.07	-0.0911
Ketoconazole		1.10	0.919
Fluconazole		1.02	0.0959
	biomass, *b*		
BDDA		1.13	0.681
Climbazole		0.76	-0.429
Ketoconazole		0.68	-1.88
Fluconazole		1.07	0.307

* < 0.05, t-test in R, version 3.1.2., package “drc”, function “compParm”.

Table S9: BDDA concentrations measured in soil as well as in *B. napus* shoot tissue at test end (day 16) in biotests without (-bios) and with (+bios) amendment of 3.8 g/kg soil d.w. and derived bioconcentration factors (BCF).

	- bios	+ bios	- bios	+ bios	- bios	+ bios	- bios	+ bios	- bios	+ bios	- bios	+ bios	- bios	+ bios	- bios	+ bios	- bios	+ bios	- bios	+ bios
Nominal soil concentration	Measured soil concentration		Shoot concentration		Recovery measured to nominal soil concentration		BCF shoot to nominal soil concentration		BCF shoot to measured soil concentration		Shoot dry weight, sum per pot		Nominal BDDA applied, sum per pot		Measured BDDA applied, sum per pot		BDDA in shoots, sum per pot		Uptake by shoots from nominal BDDA soil amount	
[mg/kg dw]	[mg/kg dw]		[mg/kg dw]		[%]						[g]		[mg]		[mg]		[µg]		[%]	
A	*B*		*C*		*D*		*E*		*F*		*G*		*H*		*I*		*J*		*K*	
					*=B/A*100*		*=C/A*		*=C/B*				*=A*0.38 kg soil dw*		*=B*0.38 kg soil dw*		*=C*G*		*=J/1000/H*100*	
56	11	18	0.126	0.060	19.7	32.4	0.0023	0.0011	0.0114	0.0033	1.267	1.327	21.3	4.20	4.20	6.89	0.1598	0.0797	0.00075	0.0019
56	16	8	0.188	0.101	27.7	14.6	0.0034	0.0018	0.0121	0.0124	1.189	1.241	21.3	5.89	5.89	3.11	0.2237	0.1254	0.00105	0.0021
56	14	9	0.130	0.147	24.8	15.9	0.0023	0.0026	0.0094	0.0165	1.332	1.241	21.3	5.27	5.27	3.39	0.1733	0.1826	0.00081	0.0035
56	14	10	0.190	0.114	24.7	17.1	0.0034	0.0020	0.0137	0.0119	1.074	1.095	21.3	5.26	5.26	3.63	0.2042	0.1249	0.00096	0.0024
112	14	21	0.267	0.178	12.3	19.0	0.0024	0.0016	0.0194	0.0084	1.076	1.062	42.6	5.24	5.24	8.09	0.2875	0.1892	0.00068	0.0036
112	33	13	0.286	0.205	29.3	11.3	0.0026	0.0018	0.0087	0.0162	0.765	1.079	42.6	12.5	12.5	4.81	0.2190	0.2214	0.00051	0.0018
112	17	17	0.398	0.179	15.3	15.3	0.0036	0.0016	0.0232	0.0105	0.917	1.317	42.6	6.53	6.53	6.51	0.3653	0.2359	0.00086	0.0036
112	16	14	0.305	0.182	14.5	12.2	0.0027	0.0016	0.0188	0.0133	0.744	0.919	42.6	6.17	6.17	5.19	0.2271	0.1674	0.00053	0.0027
224	31	56	0.563	0.343	13.8	24.9	0.0025	0.0015	0.0183	0.0062	0.371	0.377	85.1	11.7	11.7	21.2	0.2090	0.1294	0.00025	0.0011
224	27	44	0.491	0.331	12.2	19.6	0.0022	0.0015	0.0179	0.0075	0.512	0.382	85.1	10.4	10.4	16.7	0.2516	0.1265	0.00030	0.0012
224	37	45	0.778	0.275	16.5	19.9	0.0035	0.0012	0.0210	0.0062	0.313	0.415	85.1	14.1	14.1	16.9	0.2434	0.1142	0.00029	0.0008
224	37	62	0.512	0.541	16.6	27.6	0.0023	0.0024	0.0138	0.0088	0.382	0.665	85.1	14.2	14.2	23.5	0.1957	0.3601	0.00023	0.0025
448	46	62	1.913	0.901	10.3	13.9	0.0043	0.0020	0.0415	0.0145	0.128	0.209	170	17.5	17.5	23.6	0.2448	0.1883	0.00014	0.0011
448	32	77	1.110	0.467	7.1	17.2	0.0025	0.0010	0.0347	0.0061	0.043	0.162	170	12.1	12.1	29.3	0.0477	0.0757	0.00003	0.00062
448	47	67	0.992	0.556	10.5	14.9	0.0022	0.0012	0.0211	0.0083	0.265	0.104	170	17.9	17.9	25.4	0.2628	0.0579	0.00015	0.00032
448	83	70	1.257	1.329	18.6	15.7	0.0028	0.0030	0.0151	0.0189	0.069	0.159	170	31.6	31.6	26.8	0.0867	0.2113	0.00005	0.0007
896	111	112	1.755	2.087	12.4	12.5	0.0020	0.0023	0.0158	0.0187	0.16	0.122	340	42.1	42.1	42.5	0.2809	0.2546	0.00008	0.0006
896	102	93	4.318	3.266	11.4	10.4	0.0048	0.0036	0.0424	0.0351	0.094	0.135	340	38.7	38.7	35.4	0.4059	0.4409	0.00012	0.0011
896	136	105	1.881	2.462	15.2	11.7	0.0021	0.0027	0.0138	0.0235	0.025	0.05	340	51.7	51.7	39.8	0.0470	0.1231	0.00001	0.00024
896	77	81	0.836	2.299	8.6	9.0	0.0009	0.0026	0.0108	0.0285	0.202	0.102	340	29.3	29.3	30.7	0.1688	0.2345	0.00005	0.0008

BDDA = Benzyldimethyldodecylammonium (cation).

A.1.3 Quality assessment of digested sludges produced by advanced stabilization processes

Braguglia CM, Coors A, Gallipoli A, Gianico A, Guillon E, Kunkel U, Mascolo G, Richter E, Ternes TA, Tomei MC, Mininni G (2014): Environmental Science and Pollution Research 22, 7216-7235.

Author contributions:

Initials: Camilla M. Braguglia (CB), Anja Coors (AC), Andrea Gallipoli (AG), Andrea Gianico (AGI), Emmanuel Guillon (EG), Uwe Kunkel (UK), Giuseppe Mascolo (GM), Elisabeth Richter (ER), Thomas A. Ternes (TT), Maria C. Tomei (MT), Giuseppe Mininni (GMI).

Study design and planning:

ER: 5%; CB: 30%; AGI: 10%; AG: 10%; MT: 10%; GMI: 10%; other authors: 25%.

Performance of the experiments and analyses:

ER: 20%, performance of all biological tests; AG, AGI, MT: 60%, performance of sewage sludge stabilisation experiments; EG, UK, GM: 20%, chemical analysis.

Data assembling and preparing of figures:

ER: 10%, evaluation and preparation of graphics of the biological results, correlation with chemical data; CB, AG, AGI, MT: 60%, evaluation and preparation of graphics of the sewage sludge parameters and chemical results; EG, UK, GM: 30%, evaluation and preparation of graphics of the chemical results.

Data analysis and interpretation:

ER: 10%, analysis and interpretation of biological and chemical data; AC: 10%, analysis and interpretation of biological and chemical data; CB, AG, AGI, EG, UK, GM, TT, MT: 80%, analysis and interpretation of sludge parameters and chemical data.

Introduction, results and discussion:

ER: 5%; CB: 40%; AC: 20%, other authors: 35%.

Environ Sci Pollut Res
DOI 10.1007/s11356-014-3090-6

EFFECTIVE MANAGEMENT OF SEWAGE SLUDGE

Quality assessment of digested sludges produced by advanced stabilization processes

C. M. Braguglia · A. Coors · A. Gallipoli · A. Gianico · E. Guillon · U. Kunkel · G. Mascolo · E. Richter · T. A. Ternes · M. C. Tomei · G. Mininni

Received: 20 March 2014 / Accepted: 23 May 2014

Abstract The European Union (EU) Project Routes aimed to discover new routes in sludge stabilization treatments leading to high-quality digested sludge, suitable for land application. In order to investigate the impact of different enhanced sludge stabilization processes such as (a) thermophilic digestion integrated with thermal hydrolysis pretreatment (TT), (b) sonication before mesophilic/thermophilic digestion (UMT), and (c) sequential anaerobic/aerobic digestion (AA) on digested sludge quality, a broad class of conventional and emerging organic micropollutants as well as ecotoxicity was analyzed, extending the assessment beyond the parameters typically considered (i.e., stability index and heavy metals). The stability index was improved by adding aerobic posttreatment or by operating dual-stage process but not by pretreatment integration. Filterability was worsened by thermophilic digestion, either alone (TT) or coupled with mesophilic digestion (UMT). The concentrations of heavy metals, present in ranking order Zn > Cu > Pb > Cr ~ Ni > Cd > Hg, were always below the current legal requirements for use on land and were not removed during the processes. Removals of conventional and emerging organic pollutants were greatly enhanced by performing double-stage digestion (UMT and AA treatment) compared to a single-stage process as TT; the same trend was found as regards toxicity reduction. Overall, all the digested sludges exhibited toxicity to the soil bacterium *Arthrobacter globiformis* at concentrations about factor 100 higher than the usual application rate of sludge to soil in Europe. For earthworms, a safety margin of factor 30 was generally achieved for all the digested samples.

Keywords Sewage sludge · Agriculture · Enhanced stabilization processes · Organic pollutants · Heavy metals · Ecotoxicity

Responsible editor: Bingcai Pan

Electronic supplementary material The online version of this article (doi:10.1007/s11356-014-3090-6) contains supplementary material, which is available to authorized users.

C. M. Braguglia (✉) · A. Gallipoli · A. Gianico · M. C. Tomei · G. Mininni
Istituto di Ricerca sulle Acque-CNR, Area della Ricerca RM1, Monterotondo, Rome, Italy
e-mail: braguglia@irsa.cnr.it

A. Coors · E. Richter
ECT Oekotoxikologie, Flörsheim/Main, Germany

E. Guillon
ICMR UMR CNRS 7312, Université de Reims Champagne-Ardenne, Reims, France

U. Kunkel · T. A. Ternes
Federal Institute of Hydrology (BfG), Koblenz, Germany

G. Mascolo
Istituto di Ricerca sulle Acque-CNR, Bari, Italy

Published online: 07 June 2014

Introduction

The sustainable management of biosolids derived from sewage sludge processing is a complex challenge for both governmental and private organizations. Land application is an attractive output option of biosolids due to their fertilizing properties able to enhance growth of agricultural crops. On the other hand, digested sludge can contain pollutants and pathogens, which are causes of concern for human health and that can lead to accumulation of toxic substances in soils. The European Union (EU) regulated the land application of sewage sludge in a sludge directive (86/278/EEC, CEC 1986) that sets limits for several heavy metals based on the "precautionary principle" to guarantee sustainability. The 1986 Directive, however, did not set limits for pathogens and included only a few requirements for sludge treatment processes. Indeed, individual member states were allowed to adopt more stringent

standards (about, for example, heavy metals) or have included limits for pathogens and/or organic pollutants (NRC 2002). The EU Working Document (2000) proposed to include limits to certain groups of organic compounds, although it has been recognized that metal contamination of sludge is more important for affecting human health (Erhardt and Prüß 2001; Thornton et al. 2001). Yet, many organic compounds have lipophilic properties and hence transfer from wastewater to sewage sludge and may be present in residual concentrations ranging from nanogram per kilogram to milligram per kilogram values in dry solids depending on the initial amounts present, on their lipophilicity, and on the extent of degradation during wastewater and sludge treatment. Generally, the assimilation of available international research through risk assessment concludes that the examined organic compounds do not pose a risk to human health when applying biosolids to land (Eriksen et al. 2009; Clarke and Smith 2011). Nevertheless, it is recognized that continued vigilance is required to monitor and determine also the significance and implications of "emerging" contaminants for land application of sludge. To this extent, information available in the literature about determination of emerging organic pollutants in sludge are indeed helpful (Carballa et al. 2004, 2007; Mascolo et al. 2010a, b; Wick et al. 2010).

Considering the huge number of pollutants potentially present in sewage sludge, chemical monitoring data may not be sufficient to assess the environmental risk posed by sewage sludge applied to soil. Bioassays can provide useful information about the ecotoxicity of complex samples by integrating effects over their whole chemical composition (ISO 2008a). Yet, the assessment of ecotoxicity by means of bioassays is currently not included in the regulation for sewage sludge.

Agricultural use of sewage sludge on land depends largely on the possibility to improve the quality of sludge as well as to increase public confidence about sludge quality and safety. This implies the prevention of wastewater pollution at the source, e.g., by reducing the possibilities for heavy metals and organic compounds to enter wastewater treatment plants (WWTPs) and improving sludge treatment as well as ensuring the monitoring of sludge quality.

Among sludge treatment options, anaerobic digestion has been recognized to be the most appropriate stabilization technology to approach the problem of sludge reuse, also because of the added value of methane production. Anaerobic digesters are normally operated at either mesophilic (35 °C) or thermophilic (55–60 °C) conditions. In general, mesophilic anaerobic digestion of sewage sludge is applied more frequently than thermophilic one, mainly due to lower energy requirements and higher stability of the process. Nevertheless, thermophilic digestion is more efficient in terms of organic matter removal, methane production, and hygienization (Riau et al. 2010). However, the mesophilic transformation efficiency of the organic particulate matter of sludge into biogas is generally low, and consequently, the quality of final digestate is not suited to be applied on land due to residual presence of pathogen (EPA 2006).

In order to improve the anaerobic stabilization process, solids processing train can be implemented by adding pretreatment steps before digestion in order to enhance hydrolysis rate or by modifying increasing the digestion temperature up to thermophilic conditions. Indeed, a posttreatment after the mesophilic digestion could also significantly improve the quality of the final product (Lukicheva et al. 2009). In particular, the dual-stage anaerobic/aerobic digestion has been recognized as is a promising strategy to improve sludge digestibility because of the different reaction environments (Novak et al. 2003; Parravicini et al. 2008; Tomei et al. 2011), which can be advantageously exploited also for the enhanced biodegradation of particular classes of persistent organic contaminants which are aerobically biodegraded. Different strategies to enhance anaerobic digestion processes by either using sludge pretreatment methods (e.g., thermal hydrolysis or ultrasounds) or by posttreatment addition (aerobic or thermophilic digestion in dual stage) may be followed. Specifically, the investigated processes were as follows: (a) thermophilic anaerobic digestion, with or without thermal hydrolysis integration; (b) a dual-stage mesophilic/thermophilic anaerobic digestion, with or without ultrasound pretreatment; and (c) sequential anaerobic/aerobic digestion process.

The integrated sustainability assessment on both techno-economic and environmental aspects of these advanced stabilization processes was developed and reported in Bertanza et al. (2014).

The main objective of this paper was to assess the impact of these advanced stabilization processes on the quality of the final digested sludges, by assessing heavy metal concentrations, organic micropollutants, and ecotoxicity.

Of particular relevance was the assessment of some parameters not included in the EU Working Document but recognized to be important for the evaluation of the overall quality of treated sludge. Such parameters are concentrations of emerging organic contaminants, namely, quaternary ammonia compounds (QACs), pharmaceuticals, biocides and fungicides, and effects observed in ecotoxicity tests.

Materials and methods

Sludge type and characteristics

Sludge was sampled from the municipal wastewater treatment plants (WWTP) "Roma-Nord", serving about 780.000 P.E. with an average flow rate of 4.1 $m^3\ s^{-1}$. The plant includes primary clarification and activated sludge process as secondary treatment without additional nutrient removal. Waste activated sludge (WAS), collected repeatedly at different time

intervals from the oxidation tank operating at an average sludge age of 20 days, was clarified in a laboratory-scale tank in order to increase the solid content and separated from the supernatant. The obtained secondary sludge was used as feed sludge for the investigated digestion processes. Table 1 lists the average characteristics of the secondary sludge samples and mixed sludge sample (50 % primary and 50 % secondary sludge) that were used as feed in the digestion processes. The anaerobic inoculum used to start the laboratory-scale reactors of the here-investigated advanced sludge treatment processes was collected from a full-scale plant digester.

Enhanced stabilization processes

The digested sludges analyzed in this work were derived from three enhanced stabilization processes carried out in semicontinuous mode, namely, (a) thermophilic digestion, with or without thermal hydrolysis pretreatment (TT); (b) ultrasound pretreatment before dual-stage mesophilic/thermophilic digestion (UMT); and (c) sequential anaerobic/aerobic digestion (AA). Samples were prepared collecting portions of the digestion effluents during steady-state conditions. Process schemes are shown in Fig. 1, highlighting the sample code and abbreviations used further on. The first part of the code identifies the activity (e.g., thermal pretreatment + thermophilic digestion (TT)), and the second part identifies the type of the digested sample (e.g., digested untreated (DU)). A detailed description of the anaerobic treatments, including test operating conditions, is reported in the following paragraphs.

Thermophilic anaerobic digestion (with or without pretreatment by thermal hydrolysis, TT)

Digestion of sludge was carried out using two anaerobic glass digesters (volume (V)=7 L) operated in semicontinuous mode under thermophilic conditions. As control unit, one reactor was fed with untreated secondary sludge. The second reactor was fed with the same sludge but after thermal pretreatment (T=135 °C, p=312 kPa, t=20 min). Both jacketed reactors were completely mixed and maintained at the constant temperature of 55 °C. In test 1, the reactors were operated at a hydraulic retention time (HRT) of 8 days with a corresponding organic loading rate (OLR) of 1.7 g volatile solids (VS) L^{-1} day^{-1}, whereas in September, for test 2, OLR was decreased to 1.0 g VS L^{-1} day^{-1} (HRT=15 days). The digested sludge was collected daily during steady-state conditions; the resulting composite sample was stored at 4 °C. One fraction of collected fresh sample was prepared for ecotoxicological analysis, and one fraction was oven-dried (T=60 °C) before the analysis of organic and inorganic contaminants.

Regarding this activity (TT), the codes are as follows: feed untreated (TT-FU), feed after thermal pretreatment (TT-FP), digested untreated (TT-DU), and digested pretreated (TT-DP).

Table 1 Characteristics of the sludges used as feed for the investigated processes

	Secondary sludge	Mixed sludge
TS (g L^{-1})	23±5	53±2
VS (g L^{-1})	14.8±3	36±2
pH	6.5±0.3	7.1±0.4
TOC (g kg^{-1} d.w.)	340±10	370±8
Total N (g kg^{-1} d.w.)	58±5	54±4
Total S (g kg^{-1} d.w.)	15±4	10±3
Total P (g kg^{-1} d.w.)	20±2	13±1
Soluble COD (mg L^{-1})	55±5	700±50
Soluble N_{tot} (mg L^{-1})	28±6	220±30
Soluble N-NH_4 (mg L^{-1})	17±3	190±25

d.w. dry weight

Mesophilic/thermophilic anaerobic process, with or without ultrasounds pretreatment (UMT)

The experimental digesters were operated in semicontinuous mode and in series, with the sludge being fed to the mesophilic reactor once per day, and an equivalent volume of digested sludge was transferred from the mesophilic (T=37 °C) reactor to the thermophilic (T=55 °C) one. The OLR to the mesophilic reactor was fixed at 3.9 kg VS m^{-3} day^{-1} while to the successive thermophilic was 1.2 kg VS m^{-3} day^{-1}.

To assess the effect of ultrasound pretreatment, one dual-stage line was fed with untreated WAS, while the parallel line was fed with the same sludge previously pretreated. All jacketed reactors (V=7 L) were completely mixed and maintained at constant temperatures (more details in Gianico et al. (2014)). One fraction of collected fresh sample was prepared for ecotoxicological analysis, and one fraction was oven-dried (T=60 °C) before the analysis of organic and inorganic contaminants.

Regarding this activity (defined UMT), the sample codes are as follows: untreated feed (UMT-FU), pretreated feed (UMT-FP), mesophilic digested untreated (UMT-MDU), mesophilic/thermophilic digested untreated (UMT-MTDU), mesophilic digested pretreated (UMT-MDP), and mesophilic/thermophilic digested pretreated (UMT-MTDP).

Anaerobic/aerobic sequential process

The sequential anaerobic/aerobic process was tested on a laboratory-scale plant consisting of two digesters (V=7.4 L each) operated in series and in semicontinuous mode. The first reactor was operated under anaerobic conditions (T=37 °C) and HRT of 15 days, while the aerobic reactor was operated at room temperature with HRT of 12 days at intermittent aeration

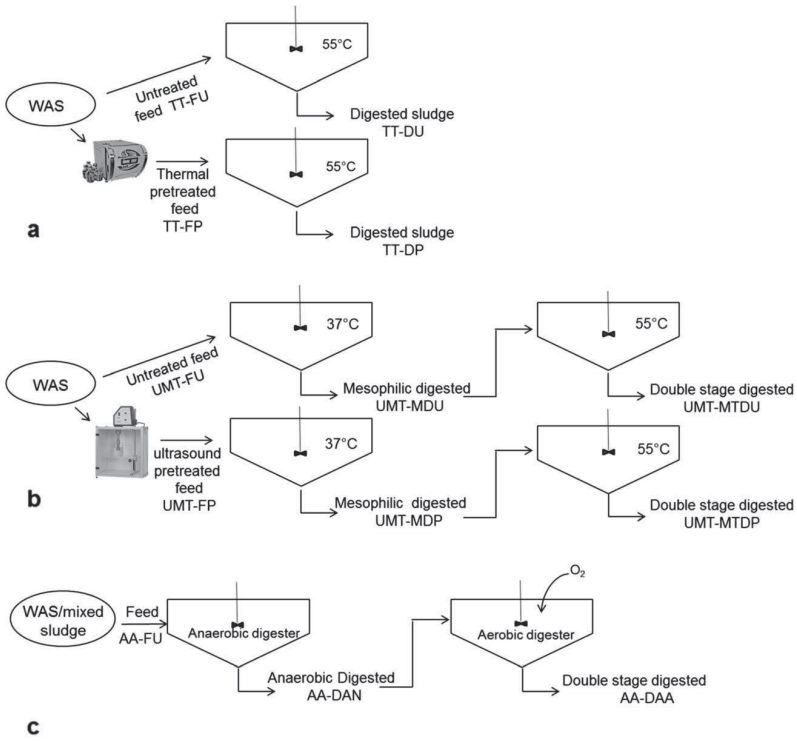


Fig. 1 Process schemes. **a** Thermophilic digestion, with thermal pretreatment. **b** Dual-stage mesophilic/thermophilic digestion with ultrasound pretreatment. **c** Sequential anaerobic/aerobic digestion and sample codes

to achieve simultaneous nitrification-denitrification conditions (more details in Tomei and Carozza (2014)).

Two series of tests were carried out after the start-up: the first test with secondary sludge and the second one with mixed sludge. Digested sludge was collected daily during stable performance periods, and the composite sample was stored at 4 °C. One fraction of collected fresh sample was prepared for ecotoxicological analysis, and the second was oven-dried (T=60 °C) before the analysis of organic and inorganic contaminants. Regarding this activity (AA), the codes are as follows: feed (AA-FU), anaerobic digested (AA-DAN), and double-stage digested (AA-DAA).

Analytical methods

Sludge composition

Total and volatile solids (TS and VS, respectively) were determined according to standard methods (APHA 1998). Total phosphorus was determined by a colorimetric method (at 680 nm) based on the formation of a blue-colored complex of P (APHA 1998). Total organic carbon (TOC), total nitrogen (N), and total sulfur (S) were determined with an elementary analyzer (Vario macro CHNS, elementar). The soluble phase was analyzed by removing the particulate sludge matter via centrifugation (10 min at 5,000 rpm). Then, the resulting centrate was filtrated through 0.45-μm pore size membrane filters. Soluble chemical oxygen demand (COD) was determined by photometric determination (COD Cell Test by Spectroquant Merck) following the EPA Method 410.4. Ammonia nitrogen was determined according to the method 4500-NH_3 C (APHA 1998).

Sludge filterability was estimated using a capillary suction time (CST) equipment supplied by Triton Electronics Ltd., UK. A stainless steel tube with an inner radius of 0.535 cm and filter paper Whatman No. 17 were used. Each sludge was analyzed five times and the results averaged, before being standardized to the TS concentration as detailed in Standard Methods (APHA 1998). In order to compensate for the different amounts of dry solids contained in the sludge, CST was "normalized" with respect to TS content (Vesilind 1988).

Heavy metal analysis

Sludge samples were accurately weighed to approximately 0.6 g directly in microwave polytetrafluoroethylene (PTFE) vessels and subjected for the digestion procedure to a 6 mL 69 % HNO_3, 2 mL 48 % HF, and 2 mL 30 % H_2O_2, in a 1:10 (g/mL) ratio, at 140 °C for 2.5 h (Sastre et al. 2002). The digestion program itself consisted of a 10-min gradual increase in temperature to 200 °C, a 15-min step at 200 °C (1,000 W; 106 Pa), and then a ventilated cooling stage. This program was chosen in agreement with the manufacturer's recommendations and earlier studies on microwave-assisted

digestion optimization. The digests were filtered through a 0.45-μm PTFE minisart SRP Sartorius filter, evaporated on a hot plate set at 60 °C, dissolved using 36 % HCl and 69 % HNO_3, and diluted to 50 mL with ultrapure water to determine total heavy metal concentrations. All the solutions were stored in polyethylene vials at 4 °C until analysis and analyzed using an ICP-OES (Thermo Fisher ICAP 6300 DUO) for seven elements (Cd, Cr, Cu, Ni, Pb, Zn, and Hg).

Conventional organic micropollutants

Extractable organic halogens (EOX) It is worth noting that in the EU Working Document, the integral parameter defined as the sum of halogenated organic compounds (AOX) is reported. According to what is reported in the literature, when measuring the total halogenated organic compounds, the term AOX is referred to adsorbable organic halogens (measured in an aqueous sample), while for a solid sample, such as a sludge sample, the term EOX is used. Therefore, from now on, the term EOX is employed. EOX were determined according to EPA Method 9023 using the Xplorer instrument (TE Instruments, Delft, The Netherlands). This method does not allow the detection of specific organic halogenated compounds but only the total amount of chlorine, bromine, and iodine due to the organic halogens of the sample. Twenty grams of dried and ground sludge was extracted with 100 mL of acetone for 30 min in an ultrasonic bath kept at 40 °C. Then, 100 mL of *n*-hexane was added and the extraction was repeated. The mixture was allowed to settle for some hours; 100 mL of organic phase is withdrawn, and 3 mL of a saturated aqueous solution of sodium sulphite was added. The organic phase was washed with 250 mL of water (two times) in order to extract the acetone solvent. The organic phase was evaporated down to 20 mL; anhydrous sodium sulfate was added, and the volume was reduced down to 5 mL. Finally, 50 μL of *n*-hexadecane was added, and the volume of the extract was reduced to 2 mL. Fifty microliters of the obtained organic extract was injected (at 15 μL min^{-1}) in the combustion chamber kept at 850 °C in an oxygen atmosphere. The HX formed was transferred to a coulometric microtitration cell where the equivalents of HX were determined. The EOX concentration is reported as micrograms of chloride per kilogram (μg Cl kg^{-1}). The detection limit of the employed procedure was determined to be 29 μg Cl kg^{-1} of dry sludge.

Linear alkylbenzene sulfonates (LAS) The experimental procedure for LAS extraction and analysis was carried out following CEN standard method for solid samples (CEN/TS 16189) extracting around 2 g oven-dried sludge samples with 10 mL methanol by mechanical shaker. Each surfactant extraction was done in duplicate. The separation of the four different LAS homologues (C10, C11, C12, and C13) was performed with a Dionex HPLC equipped with a low-pressure gradient pump with integrated vacuum degasser, autosampler, and an Acclaim Surfactant (Dionex) column 5 μm 120 Å (2.1×150 mm). The isocratic mobile phase was acetonitrile/100 mM ammonium acetate (65:35 *v*/*v*) flowing at 0.2 mL min^{-1}. LAS determination was obtained with a single quadrupole mass detector AB-Sciex API 165 equipped with a Turbo IonSpray source, operating in negative ionization mode. LAS identification and quantification were performed in full-scan acquisition and in selected ion monitoring (SIM) mode, respectively.

The identification of LAS homologues was based on the retention time of the homologues and the isomers of each homologue. LAS target ions were used for identification of the different homologues (Online Resource 1).

To correct the losses during the analytical procedure and to calculate the concentration of the analytes, 4-octylbenzenesulfonate (C8-LAS) was added to the sludge samples before extraction as internal standard. The repeatability of the instrumental method was tested by running LAS mixture standards at two concentration levels for ten times; the variance was 4.8 and 5.3 %, respectively.

Nonylphenol ethoxylates (NPEOs) Determinations of alkylphenols and alkylphenol ethoxylates were performed according to CEN/TC CSS99040 method with a modification about the chromatographic analysis. In fact, liquid chromatography/mass spectrometry was used instead of gas chromatography/mass spectrometry according to a published procedure (Jahnke et al. 2004). Specifically, the extraction and cleanup were performed using pressurized solvent extraction (PLE) by an ASE 200 instrument (Thermo Fisher Scientific). Briefly, 0.67 g of dried and ground sludge was mixed with neutral alumina and copper at the ratio of 1:3:3. The mixture was placed in 66-mL extraction cells between two layers of diatomaceous earth. The extraction was carried out by acetone/*n*-hexane 1:1 (three cycles at 60 °C). The obtained extract was evaporated down to 0.5 mL. The extract was then analyzed by UPLC/MS-MS (Waters Acquity interfaced to a AB-Sciex API 5000), and for the quantitation, product ions listed in Online Resource 2 were employed. The detection limit of the employed procedure was determined to be between 0.8 and 3.7 μg kg^{-1} dry sludge depending of the specific pollutants (see Online Resource 2).

Polycyclic aromatic hydrocarbons (PAHs) Determinations of PAHs were performed employing EPA Methods 3545 and 8275 for extraction and analysis, respectively. Specifically, the PAH extraction and cleanup were performed by PLE by an ASE 300 instrument (Thermo Fisher Scientific) as described in the Thermo technical note 1025. Briefly, 5 g of dried sludge was mixed with 10 g of diatomaceous earth, and then the obtained mixture was placed in 66-mL ASE cells after a layer of 20 g of acidic alumina and a filter. The

extraction was carried out by dichloromethane (four cycles at 125 °C, 1,500 psi). The obtained extract was evaporated down to 1 mL. The extract was then analyzed by GC/MS in full scan, and for the quantitation, the molecular ions (see Online Resource 3) were employed. The detection limit of the employed procedure was determined to be between 5 and 12.5 μg kg^{-1} dry sludge depending of the specific pollutants (see Online Resource 3).

Polychlorinated biphenyls (PCBs) PCBs were determined on the basis of CEN 15308 method. The quantified congeners were those having numbers 28, 52, 101, 118, 138, 153, and 180, and the labelled congener 13C-12PCB-104 was used as an internal standard. The PCB extraction and cleanup were performed using PLE by an ASE 200 instrument (Thermo Fisher Scientific) as described in the technical note 210. Briefly, 2 g of dried sludge were mixed with anhydrous sodium sulfate and diatomaceous earth, and then the obtained mixture was placed in 66-mL ASE cells after a layer of acid-impregnated silica gel (10 g). The cell was then filled with diatomaceous earth. The extraction was carried out by *n*-hexane (two cycles at 100 °C, 1,500 psi). The obtained extract was evaporated down to 1 mL. The extract was then analyzed by GC/MS-MS isolating the precursor ions, fragmenting them, and finally by acquiring the full mass spectrum in the scan range listed in Online Resource 4. For the quantitation of PCB congeners, the specific product ions of each congener were extracted from the full product ion mass spectrum. Instead of a simple GC/MS as specified in the CEN 15308 method, analysis by GC/MS-MS was employed in order to improve the detection limit which was 0.5 μg kg^{-1} dry sludge.

Phthalates Phthalates were determined using EPA Methods 3545 and 8270 for extraction and analysis, respectively. Specifically, phthalate extraction and cleanup were performed by PLE by an ASE 300 instrument (Thermo Fisher Scientific). Briefly, 5 g of dried sludge was mixed with diatomaceous earth; then, the obtained mixture was placed in 66-mL ASE cells. The extraction was carried out by acetone/*n*-hexane 1:1 (two cycles at 125 °C, 1,500 psi). The obtained extract was evaporated down to 1 mL. The extract was then analyzed by GC/MS in full scan, and for the quantitation, the molecular ions listed in Online Resource 5 were employed. The detection limit of the employed procedure was determined to be between 5 and 50 μg kg^{-1} dry sludge depending on the specific pollutants (see Online Resource 5).

Emerging organic micropollutants

Quaternary ammonia compounds Twenty-one quaternary ammonia compounds (QACs) were analyzed (see Online Resource 6). The dried sludges were first ground and then sample extraction was conducted by PLE with an ASE 350 system (Thermo Fisher Scientific). The sludge (0.2 g) was filled into 10-mL extraction cells, and isotopically labelled internal standards were added (750 ng of each surrogate standard). Cells were filled with sea sand and sludge was mixed with the sea sand. PLE was accomplished by four extraction cycles with methanol at a pressure of 100 bar and a temperature of 80 °C. Subsequently, the extracts were diluted with MeOH to a volume of 30 mL. Approximately 1 mL of the diluted extract was transferred into an HPLC vial and subsequently measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Determination of QACs was done by a LC-(ESI)MS/MS system consisting of a binary LC pump (Agilent 1200) and a tandem mass spectrometer (API 4000, AB Sciex, Darmstadt, Germany). Separation was achieved using a binary gradient of water (10 mM ammonium formate (NH_4FA) + 0.1 % formic acid (FA)) and methanol (10 mM NH_4FA + 0.1 % FA) on a Hydro-RP column (150×3 mm, 4 μm, Phenomenex); the MS was operated in the positive ionization mode, and the transitions of the QACs were monitored using a scheduled multiple reaction monitoring (SMRM) mode. The injection volume was 10 μL and the column temperature was set to 25 °C. The limits of quantification for the individual QACs ranged from to 50 to 350 μg kg^{-1} (Online Resource 6).

Pharmaceuticals, biocides, and fungicides Eleven pharmaceuticals and 13 biocides and fungicides were determined in the sludge samples (see Online Resource 7 for individual compound list). Dried sludge was ground for the analysis, and sample extraction was conducted by PLE with an ASE 200 system (Thermo Fisher Scientific). To this end, 0.2 g of ground sludge was filled into extraction cells (size=22 mL) and isotopically labelled internal standards were added (10 ng of each surrogate standard). Cells were filled up with sea sand and sludge and sand were thoroughly mixed. PLE was accomplished by four extraction cycles with a mixture of methanol/water (50:50, *v*/*v*) at a pressure of 100 bar and a temperature of 80 °C. The extracts were diluted with ultrapure water to a total volume of 50 mL; approximately 1 mL of the diluted extract was transferred into an HPLC vial and subsequently measured by LC-MS/MS.

Determination of pharmaceuticals and biocides was done by a LC-(ESI)MS/MS system consisting of a binary LC pump (Agilent 1260) and a tandem mass spectrometer (QTRAP 5500, AB Sciex). Quantification was done using isotopic dilution methods. Separation was achieved using a binary gradient of water (10 mM NH_4FA + 0.1 % FA) and methanol (0.1 % FA) on a Zorbax-Eclipse column (XDB C18, 150× 2.1 mm, 4 μm, Agilent); the MS was operated simultaneously in both positive and negative ionization modes using SMRM mode. The injection volume was 40 μL and the column temperature was set to 40 °C. The limits of quantification for

the individual pharmaceuticals, biocides, and fungicides ranged from to 2.5 to 25 μg kg^{-1} (Online Resource 7).

Ecotoxicological tests

The selection of biotests and the ecotoxicologial testing approach employed in the present investigation followed the international guideline for the ecotoxicological testing of waste (CEN 2005) together with recommendations from earlier studies on sludge and wastes that indicated which test systems would be most sensitive for these kinds of materials (Moser and Roembke 2009). The set of applied test systems, however, was driven by the limited amount of sewage sludge available for testing. Selected terrestrial biotests were (a) the test for inhibition of enzyme activity in the soil bacterium *Arthrobacter globiformis* and (b) the test for avoidance behavior of the earthworm *Eisenia fetida*.

In the environmental risk assessment of chemicals that is based on ecotoxicological laboratory standard tests, safety factors between 10 and 1,000 are applied, with the exact value depending on the type of chemical and the amount of available information (i.e., test results for different trophic levels and acute as well as chronic endpoints). The safety factor in chemicals' environmental risk assessment serves to cover the uncertainty due to the extrapolation from laboratory tests to the real-world situation and from standard test species to organisms potentially exposed in the environment. While in the context of the quality assessment of sludge, no such safety factors are legally established, a similar safety margin as in the environmental risk assessment of chemicals appears warranted. Therefore, the ecotoxicological results obtained in the present study are compared to the application rates of sludge to agricultural land in order to determine the resulting safety margin. Both the usual application rate in Europe, i.e., 2 t ha^{-1} (EC 2010), and the maximum allowed application rate in Ontario, Canada, i.e., 22 t ha^{-1} (OR 2009), are here considered. Assuming a plowing depth of 20 cm and a soil bulk density of 1.3 g cm^{-3}, these application rates result in 0.8 g sludge kg^{-1} soil (Europe) and 8.5 g sludge kg^{-1} soil (Ontario).

Oven-dried samples of feed and digested sludges derived from the different advanced treatment processes were tested by two terrestrial ecotoxicity tests. As an external reference for sludge treated by standard methods, biosolids, officially deemed fit for application on land (Cogger et al. 2006), were included in the testing program. These biosolids (anaerobically digested sewage sludge) were obtained from a municipal sewage treatment plant in Waterloo, Ontario, Canada, and represent treated sewage sludge that is routinely applied to agricultural land in Ontario. Tests with *A. globiformis* were conducted according to the international draft guideline ISO 18187 (2012). The endpoint of the *A. globiformis* test is the inhibition of dehydrogenase, a key enzyme of many organisms. A dilution series with five dilutions (between 0.1 % and up to 50 % sludge added to the substrate quartz sand) was tested to estimate the median effect concentration (EC_{50} in gram sludge dry weight per kilogram quartz sand dry weight). The maximum tested sludge concentration was 250 g sludge kg^{-1} substrate (in two cases 500 g sludge kg^{-1} substrate). Dilutions and control substrate were moistened to 33 % relative moisture using distilled water and were homogenized in small glass beakers. Afterwards, they were distributed into 24-well plates at 0.6 g wet weight per well with four replicates per treatment. After adding 0.6 mL distilled water to each well, the plates were pasteurized at about 60 °C in a water bath twice for 15 min. After cooling the bacterial inoculum, or in the case of the blanks, only the nutrient solution was added at 0.4 mL per well. Well plates were then incubated for 2 h at 30±2 °C on a shaker in the dark. The fluorescence dye resazurin (CAS number 62758-13-8; dissolved in phosphate buffer at 45 g L^{-1}) was then added at 0.8 mL per well. Caused by the dehydrogenase activity of *A. globiformis*, resazurin is transformed into resorufin, which was measured using the spectral fluorometer (Tecan multiplate reader, Tecan Group Ltd., Männedorf, Switzerland, exCitation at 535 nm and emission at 590 nm) every 15 min for 1 h. The slope of fluorescence increase between 15 and 45 min of each dilution was compared to that of the control and was expressed as % inhibition. EC_{50} and their 95 % confidence intervals (CIs) were estimated by fitting a concentration curve to the data using the probit model (Finney 1978).

Earthworm avoidance tests were performed with *E. fetida* according to the ISO 17512-1 guideline (ISO 2008b). Due to the limited amount of available sludge, it was not possible to test a full range of dosages suitable to derive an EC_{50} estimate for avoidance but only to conduct tests at very few different dosages. Sludge samples were applied at maximum with 25 g dry sludge kg^{-1} soil d.w. in the test. This resembled about 3-fold and 30-fold greater application rates than those assumed for Ontario and Europe, respectively. The appropriate amount of sludge was mixed into artificial soil (OECD 1984) and moistened with distilled water to 40–60 % of the maximum water holding capacity (WHC_{max}). The sludge-soil mixtures were tested against control soil (i.e., 100 % of the artificial soil). A second control was included that contained a corresponding addition of peat (i.e., 25 g kg^{-1}), to take into account an eventual preference of earthworms for high organic soils as resulting from addition of sludge. While filling the soil into the test vessels (plastic boxes, volume 0.75 L), a partition wall was inserted at the middle to fill one half each with control soil and the respective test soil (about 265 g fresh weight each). For each treatment, there were five replicates with 10 earthworms each (fresh weight between 300 and 600 mg) that were placed at the border of the two soils in the vessel. Afterwards, the test vessels were closed with a

perforated lid and were kept in a climate chamber (20±2 °C) for 48 h at a light intensity between 400 and 800 lux and a 16:8 h light cycle. After 48 h, the partition wall was inserted again in the middle of the vessels and the worms on each side were counted. The results were expressed as percentage of animals avoiding the sludge-amended soil. According to the guideline, avoidance by ≥80 % is defined as the threshold value that indicates a limited habitat function of the test soil.

Results and discussion

Feed sludge characteristics

Main characteristics and contaminant concentrations of the sewage sludge used as feed for the investigated processes are listed in Tables 1, 2, and 3. It must be pointed out that all the digestion tests, carried out during 2 years of experimentation, were performed by feeding secondary sludge from the same source (WWTP Roma-Nord), collected at different times, while one test of the AA process was performed by feeding mixed sludge. Accordingly, the pollutant concentrations related to the secondary sludge are expressed as ranges, while those related to the mixed sludge of the AA process as average of the analytical replicates carried out on the same sample. Individual concentrations detected in the sludges used as feed for the three different processes are reported in the Online Resource.

The high sludge age in the aeration basin caused the VS content of both sludges to be quite low, namely, around 64 and 68 % for the secondary and the mixed sludge, respectively. Nutrient content of the secondary sludge was 5.8 and 2 % of TS for nitrogen and phosphorus, respectively, being typical values for biological sludge (Metcalf and Eddy 2003). Mixed sludge was slightly poorer in nutrients, especially phosphorus, but richer in soluble organic compounds as COD and ammonia (Table 1).

Despite the long sampling period and potential for seasonal effects, little variability was observed for heavy metal concentration (Gianico et al. 2013a). Moreover, heavy metal concentrations in the mixed sludge are in the typical concentration range for secondary sludge. Zinc was always the most abundant metal (ranging from 442 to 764 mg kg^{-1}), while Cd was present at the lowest concentration (0.8–1.2 mg kg^{-1}). The next most abundant metals were Cu, Pb, Cr, and Ni.

On the contrary, significant variability was evidenced for organic micropollutant concentration (Table 3), probably due to seasonality affecting the source of contamination.

It must be pointed out that the secondary sludge is typically less polluted than the corresponding primary sludge, since hydrophobic pollutants tend to adsorb during primary sedimentation. Unfortunately, secondary sludge contamination is scarcely investigated in literature, and comparison is mostly with raw mixed sludge. LAS and phthalates are the most abundant organic contaminants present in sewage sludge, as reported in the survey of Abad et al. (2005) together with NPEOs.

NPEOs were detected in concentrations 1–4 mg kg^{-1}, with NPEO1 and NPEO2 predominant species, significantly lower with respect to the values reported by Abad et al. (2005) for municipal raw sludge in Spain. Other surveys reported concentrations in Norwegian sewage sludges in the range 22–650 mg kg^{-1} (Paulsrud et al. 1998).

Concentrations of anionic surfactants found in secondary sludge are in the range 115 and 630 mg kg^{-1}, reaching a concentration of 1,550 mg kg^{-1} in the case of mixed sludge. In literature, LAS concentrations in sludge mostly refer to stabilized sludge and few data about untreated sludge are available (Carballa et al. 2009). Jones and Nothcott (2000) reported LAS concentration of Spanish mixed sludge in the range 400–700 mg kg^{-1}, while Aparicio et al. (2009) reported a mean concentration of anionic surfactants in secondary sludges around 275 mg kg^{-1}, comparable with the values of the secondary sludge investigated in this study.

PAH concentration ranged between 1.7 and 3.6 mg kg^{-1} for the secondary sludge, while the mixed sludge presented a concentration of 3.2 mg kg^{-1}. Madsen et al. (1997) detected PAH concentrations (sum of nine PAHs listed in the EU Working Document) in Danish sludges generally below

Table 2 Heavy metal concentration (mg kg^{-1}) of the sludges used as feed for the investigated processes

	Secondary sludge	Mixed sludge
Cd	0.8–1.2	1.0
Cr	20–29	26
Cu	144–241	234
Ni	14–30	38
Pb	58–94	94
Zn	442–764	720
Hg	n.d.–0.8	n.d.

d.w. dry weight, *n.d.* not determined

Table 3 Conventional and emerging organic pollutant concentration (μg kg^{-1}) of the sludge used as feed for the investigated processes

	Secondary sludge	Mixed sludge
EOX	4,650–12,000	4,500±421
NPEs	970–3,990	4,400±450
LASs	115,000–630,000	1,550,000±160,000
PAHs	1,730–3,630	3,170±210
PCBs	11–22	42±16
Phthalates	25,000–86,100	57,600±6,100
QACs	26,000–49,000	130,000±6,300
Pharmaceuticals	100–160	120±10
Biocides and fungicides	1,400–3,600	3,600±460

3 mg kg^{-1}. However, the results presented here are difficult to compare with those already published since most of them are not homogeneous with respect to the number of PAH compounds they represent.

PCBs reported in Table 3 correspond to the sum of PCB #28, #52, #101, #118, #138, #153, and #180, which are routinely measured as representatives of the PCB fraction. All the samples presented concentrations lower than the 0.8-mg kg^{-1} limit (CEC 2000). The values found in these samples are 0.011–0.022 mg kg^{-1} in the secondary sludge and 0.042 mg kg^{-1} in the mixed sludge. Spanish sludge presented a PCB concentration in the range 0.003–0.06 mg kg^{-1}, while Berset and Holzer (1996) found PCB concentrations of 0.043–0.55 mg kg^{-1} in samples collected from 19 sewage treatments in Switzerland. Similarly, a recent study (Blanchard et al. 2004) reported PCB concentrations ranging from 0.07 to 0.65 mg kg^{-1} in sludge samples taken in a WWTP in France, where the secondary sludge presented a concentration of 0.149 mg kg^{-1}. Phthalates (di(2-ethylhexyl)phthalate, di-*n*-butylphthalate, and benzylbutylphthalate) range between 0.19 and 154 mg kg^{-1} (Smith and Riddell-Black 2007). In the investigated secondary sludge, phthalates accounted for 25–86 mg kg^{-1} with a value of 57 mg kg^{-1} for the mixed sludge.

Overall, for conventional organic micropollutants, the measured concentrations are in the same range of those already reported in the literature. For LAS, PAHs, and phthalates in few samples, the concentrations found were higher than those listed in the EU Working Document (CEC 2000).

QACs are present in raw wastewater in high concentrations up to few hundred milligrams per liter (Kreuzinger et al. 2007) and are almost completely removed during wastewater treatment by sorption to excess sludge and biotransformation processes (Clara et al. 2007). The concentrations in both sludges (Table 3) are in good agreement with previous reported data (Martinez-Carballo et al. 2007). Higher concentrations in mixed sludge can be explained by the high sorption coefficients (log K_d ~3–5) of the QACs and removal during primary sedimentation (Ismail et al. 2010).

As for pharmaceuticals, not all individual substances are discussed in detail. Carbamazepine (anticonvulsant) is one of the most frequently detected pharmaceuticals in environmental and WWTP samples. At WWTP Roma-Nord, concentrations of carbamazepine in both mixed and secondary sludges were relatively low (10–20 μg kg^{-1}). However, these values are in good agreement to literature values (e.g., 120 μg kg^{-1} in Barron et al. 2008 or 10–20 μg kg^{-1} in Sponberg and Witter Spongberg and Witter 2008). In accordance, concentrations for the analgesic diclofenac of up to approximately 50 μg kg^{-1} are in the same concentration ranges as those previously reported (Spongberg and Witter 2008). Most pharmaceuticals are relatively hydrophilic and hardly removed during primary treatment (Carballa et al. 2004). Therefore, concentrations in mixed and secondary sludges were almost equal.

Investigated biocides and fungicides were also in similar concentration ranges in both mixed and secondary sludges (Table 3). Moreover, the concentrations of the commonly detected fungicide climbazole (secondary sludge 300–600 μg kg^{-1}, mixed sludge 860±170 μg kg^{-1}) and the disinfectant triclosan (secondary sludge 700–2,200 μg kg^{-1}, mixed sludge 2,400±250 μg kg^{-1}) are in the lower range of literature data derived for mixed sludge (Radjenovic et al. 2009; Wick et al. 2010).

Enhanced stabilization processes

The suitability of biosolids for land application can be determined by biological, chemical, and physical analyses, as well as by ecotoxicological tests (Wang et al. 2009).

The composition of treated sludges depends predominantly on raw wastewater composition and applied treatment processes. One of the most important properties is the VS content that provides an estimation of the readily decomposable organic matter, in order to control potential odor problems at land application sites. One of the goals of anaerobic digestion processes, in particular, the advanced ones, is the reduction of VS content and, thus, the odor potential. Generally, VS/TS ratios below 0.6 and VS removal rate above 40 % indicate an achieved stabilization, and therefore, risks of further putrescibility and odor formation are neglectable.

Sludge quality was also assessed in terms of heavy metals (several metals are regulated in the sludge Directive 86/278) and organic (conventional and emerging) contaminants.

Because of the biological and chemical stability of heavy metals, these potentially toxic elements are conserved and retained in the sludge during the digestion process (Thornton et al. 2001). For this reason, if process and sampling procedure are properly performed, metal concentrations should increase in direct proportion to the loss of solids.

Although organic pollutants are not included in the aforementioned directive, further assessment of several specific organic compounds is conducted here as it has been recommended by the National Research Council (2002). Removal rates of organic pollutants have been calculated considering the residual concentration in the final digested sludge with respect to that measured in the respective individual feed sludge, normalized for the reduction of organic matter.

Last but not least, results on pathogen quantification, a stringent requirement to evaluate the hygienic quality of these sludges, are reported and deeply discussed in Levantesi et al. (2014).

Thermophilic anaerobic digestion (with thermal hydrolysis pretreatment)

The stability index of the digested sludge, evaluated by means of VS/TS ratio (Table 4), was neither affected by the thermal

Table 4 Characteristics of digested sludge and supernatant derived from the thermophilic digestion of untreated or thermally pretreated sludge

	Test 1	
	TT-DU	TT-DP
VS/TS (–)	0.54	0.53
Soluble COD (mg L^{-1})	697±45	1132±102
Soluble N-NH_4 (mg L^{-1})	939±54	1009±78
CST (s)	384	574
CST (s L g^{-1})	25±1.8	39±3
	Test 2	
	TT-DU	TT-DP
VS/TS (–)	0.54	0.56
Soluble COD (mg L^{-1})	621±54	995±87
Soluble N-NH_4 (mg L^{-1})	767±45	757±64
CST (s)	432	478
CST (s L g^{-1})	30±1.5	35±3.1

pretreatment nor by the organic load variation (from 1 to 1.7 g VS L^{-1} d^{-1}). The integration of the thermal pretreatment worsened the quality of the supernatants in terms of organic substances (measured as soluble COD), while it did not affect the final ammonia level. Although ammonia concentration increased after thermal hydrolysis (data reported in Gianico et al. 2013b), organic loading rate appeared to be the most significant parameter affecting ammonia concentration in anaerobic supernatants, in agreement with the findings of Wilson and Novak (2009).

Moreover, for the sludge derived from pretreated feed digestion, deterioration of filterability was observed in both tests, and CST value increased significantly (Table 4). Sludge filterability is influenced by solids concentration and VS/TS, as are particle charge and floc density (Yukseler et al. 2007). The colloidal polymers entrapped in the flocs were intensely released due to the thermal pretreatment and not completely removed by the successive degradation process, worsening the final sludge filterability. Typical digested biosolids collected from thickeners prior to polymer addition and dewatering at a full-scale plant as evidenced by a normalized CST value between 25 and 30 s L g^{-1} TS (Taylor and Elliott 2013), while CST values for unconditioned sludges range between 100 and 300 s (USEPA-United States Environmental Protection Agency 1987).

The concentrations of the heavy metals determined in the digested sludges by feeding either untreated or pretreated feed are listed in Table 5. Due to the weight loss during anaerobic digestion, whereby biodegradable matter is decomposed to biogas (Langenbach et al. 1994; Tchobanoglous and Burton 1996), the heavy metal concentrations in the digested samples were higher with respect to those of the feed (reported in Online Resource 9) but noticeably lower with respect to

Table 5 Concentrations (mg kg^{-1}) of heavy metals in the digested sludges of TT process

	Test 1		Test 2	
	TT-DU	TT-DP	TT-DU	TT-DP
Cd	1.12±0.01	1.21±0.01	1.36±0.01	1.23±0.01
Cr	42.3±0.2	44.3±0.2	37.1±0.1	35.0±0.3
Cu	310±1	319±1	301±1	311±1
Ni	24.6±0.1	26.7±0.3	59.4±0.2	56.9±0.4
Pb	134±1	136±1	122±0.4	123±1
Zn	765±4	762±4	943±2	966±2
Hg	1.49±0.05	1.22±0.06	1.51±0.05	1.20±0.04

European limits reported in Directive 86/278 (see Online Resource 8), indicating the suitability of these sludges for agricultural use.

Mesophilic/thermophilic dual stage anaerobic process (with ultrasound pretreatment)

The stability index of the final digested sludge was not affected by the ultrasound pretreatment, while a significant improvement after the thermophilic stage was evidenced (Table 6). The integration of the ultrasound pretreatment did not deteriorate the quality of the supernatants in terms of organic substances and ammonia. However, during the thermophilic stage, the degradation of particulate organic matter improved significantly, producing a supernatant rich in soluble COD and ammonia, which requires further treatment (e.g., recycled to the head of the plant).

For the sludge derived from sonicated pretreated feed digestion, slight deterioration of filterability with respect to the untreated one was observed only after the mesophilic stage, while at the end of the entire process, sludge filterability was comparable.

Evidently, the significant floc disintegration due to sonication and mesophilic fermentation prior to thermophilic digestion was mitigated, and CST values were brought down to a narrow range following digestion in which the control and pretreated sludge had almost equal CST values.

The order of total metal content in the mesophilic/thermophilic digested sludge (Table 7) was Zn > Cu > Pb > Cr > Ni following the same order of the feed sludge (see Online Resource 10). As expected, no metal removal occurred and the remaining concentrations were lower than the EU limits.

Anaerobic/aerobic sequential process

Table 8 shows the main parameters characterizing the anaerobically/aerobically digested sludges in the two experimental periods. A good stability improvement due to the

Table 6 Characteristics of digested sludge and supernatant from the dual-stage process UMT

	Untreated		Pretreated	
	MDU	MTDU	MDP	MTDP
VS/TS (–)	0.53±0.02	0.47±0.01	0.53±0.03	0.47±0.01
Soluble COD (mg L^{-1})	210±18	930±88	252±19	871±69
Soluble N-NH4 (mg L^{-1})	553±42	780±67	614±57	921±89
CST (s)	148±10	350±20	208±14	378±30
CST (s L g^{-1})	6.7±0.2	20±0.8	9.9±0.7	21±1

aerobic posttreatment with a decrease of the VS/TS ratio from 0.59 to 0.55 for secondary sludge and from 0.60 to 0.57 for the mixed sludge was observed.

Soluble COD removal efficiencies in the aerobic stage were in the range of 17–50 %, but it is worth noting that the soluble COD pattern was characterized by high variability (data not shown) due to the variability of the feed sludge and of the performance of the biological system. High COD concentrations in the final stream were derived both from the influent load and from the marked VS removal.

The aerobic posttreatment permitted to ameliorate the sludge dewaterability, in particular, for mixed sludge.

Due to efficient nitrification during the aerobic step, in particular, for mixed sludge, the ammonia content of the supernatant was around 200 mg L^{-1} for the secondary sludge and decreased to ~40 mg L^{-1} in the case of the mixed sludge. Detailed presentation and data analysis are reported in Tomei and Carozza (2014).

As already discussed for the other treatments, also in the case of this sequential AA process, the concentration of heavy metals detected in digested samples (Table 9) represents the concentration of the feed (reported in Online Resource 11) normalized with respect to the residual mass after solid removal, highlighting that no removal occurred. But again, as in the case of TT and UMT samples, the heavy metal concentration in both digested sludges remained lower than the EU limits, allowing their use on land.

Table 7 Concentration (mg kg^{-1}) of heavy metals in the digested sludges of UMT

	Untreated		Pretreated	
	MDU	MTDU	MDP	MTDP
Cd	0.60±0.01	1.82±0.01	0.52±0.01	1.12±0.01
Cr	24±0.1	33.5±0.1	21.6±0.1	28.7±0.1
Cu	178±1	210±1	176±1	214±1
Ni	17.50±0.02	20.2±0.1	14.8±0.1	19.2±0.1
Pb	74±1	92±1	61.2±0.4	87±1
Zn	576±1	725±2	557±3	678±3
Hg	0.58±0.02	0.69±0.03	0.61±0.02	0.82±0.03

Fate of conventional organic micropollutants

The conventional organic micropollutants investigated during the advanced anaerobic sludge treatments were LAS, nonionic surfactants, PAHs, PCBs, phthalates, and EOX. All these parameters are included in the third draft (CEC 2000) of the EU Working Document on Sludge (see Online Resource 12). The obtained results are depicted in Figs. 2, 3, 4, 5, 6, and 7 where the measured concentration in the digested sludge derived from each investigated treatment (TT, UMT, and AA) was always compared to the normalized feed concentration. The normalized feed concentration was defined as the pollutant concentration determined in the feed sludge (reported in Online Resources 13, 14, and 15) normalized with respect to the residual mass after digestion. Hence, the normalized feed concentration represents the theoretical pollutant concentration in the digested sample if no degradation and volatilization of the pollutant had occurred. By means of this comparison, the effective elimination of pollutants during the process can be assessed.

The removal of organic micropollutants during anaerobic digestion may impact the suitability of digested sludge for agricultural disposal and is hence an important issue. Their removal, including biological and abiotic mechanisms, considerably varies between the various published studies about anaerobic digestion of contaminated sludge. It is difficult to generalize, but removal yields by anaerobic digestion of sludge may be typically in the range 20–40 % (Hijaz 2007; Barret et al. 2010). Operational parameters such as retention

Table 8 Characteristics of digested sludge and supernatant derived from the sequential process AA

	Secondary sludge		Mixed sludge	
	DAN	DAA	DAN	DAA
VS/TS (–)	0.59	0.55	0.60	0.57
Soluble COD (mg L^{-1})	724±160	548±180	734±120	587±151
Soluble N-NH4 (mg L^{-1})	852±52	135±76	896±118	42±25
CST (s)	470±45	350±60	538±78	253±72
CST (s L g^{-1})	20.4±3.1	19.4±3.4	16.4±2.2	14.0±4.9

From Tomei and Carozza (2014)

Table 9 Concentration (mg kg^{-1}) of heavy metals in the digested sludges of AA process

	Secondary sludge	Mixed sludge
	AA-DAA	AA-DAA
Cd	1.66±0.04	2.2±0.01
Cr	36.0±0.2	33.0±0.1
Cu	395±4	488±1
Ni	63±1	53±0.2
Pb	160±1	148±0.5
Zn	1,528±6	1,154±3
Hg	1.45±0.04	1.60±0.01

time and temperature may influence the biodegradation of organic pollutants such as phthalates and nonylphenol ethoxylates (Trably et al. 2003; El-Hadj et al. 2006; Patureau et al. 2008; Carballa et al. 2009). Moreover, bioavailability and co-metabolism were identified as the hypothetical mechanisms driving micropollutant biodegradation (Dionisi et al. 2006; Patureau and Trably 2006).

Due to their hydrophobic character, LAS are strongly associated with sludge-suspended solids and their fate is mainly determined by sorption to the sludge and by biodegradation, mainly in aerobic conditions. In fact, as reported elsewhere (Carballa et al. 2009; Díaz-Cruz et al. 2009; Smith 2009), LAS concentration in anaerobically digested sludge was found to be generally higher than in aerobically stabilized sludge. In accordance, the highest elimination, around 90 %, was observed during the AA process (see Fig. 2). Moreover, LAS were efficiently removed (Fig. 2) during the UMT process (70 % for the untreated sludge and 55 % for the pretreated one), mainly during the first mesophilic stage. LAS were not removed and accumulated during both tests of the TT process.

This result may suggest that the extraction of LAS from the sludge matrix to the liquid phase, and consequently their availability to biodegradation, was higher under mesophilic

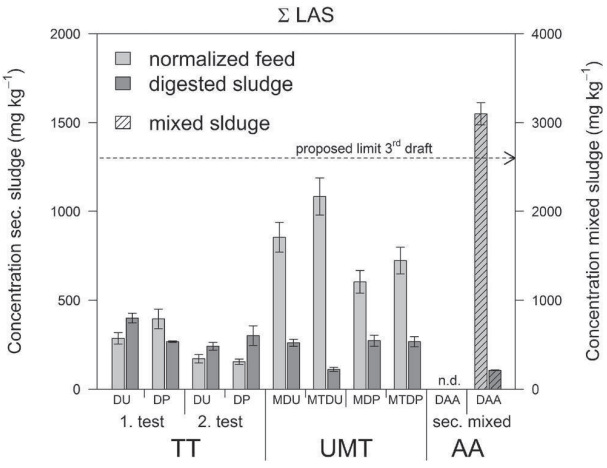


Fig. 2 LAS concentration in the digested sludge samples derived from the different advanced stabilization processes

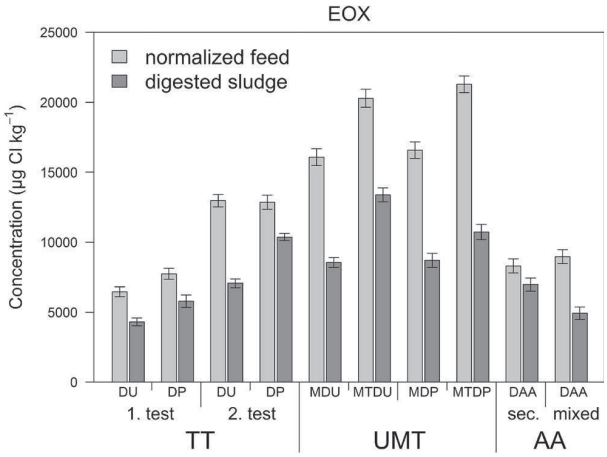


Fig. 3 EOX concentration in the digested sludge samples derived from the different advanced stabilization processes. The suggested limit (500 mg Cl kg^{-1}) of the EU Working Document is far above the highest value of the concentration axis

conditions than under thermophilic ones, as already found by El-Hadj (2006). In the mixed feed, LAS concentration normalized with respect to the hypothetical residual mass (Fig. 2) raised up to 3,100 mg kg^{-1} (Fig. 2), exceeding the threshold value recommended by the EU Working Document (CEC 2000), but the concentration was significantly reduced due to the removal that occurred during the AA treatment, thus allowing possible land application of the digested sludge.

During the thermophilic digestion (TT) of the untreated sludge, accumulation of the anionic surfactants in the digested effluents was found independently on the applied OLR, suggesting a release of some aged LAS into the extractable phase by means of the anaerobic digestion process, influencing negatively the removal efficiency, as also reported elsewhere (Gallipoli et al. 2014; Patureau et al. 2008; Trably et al. 2003).

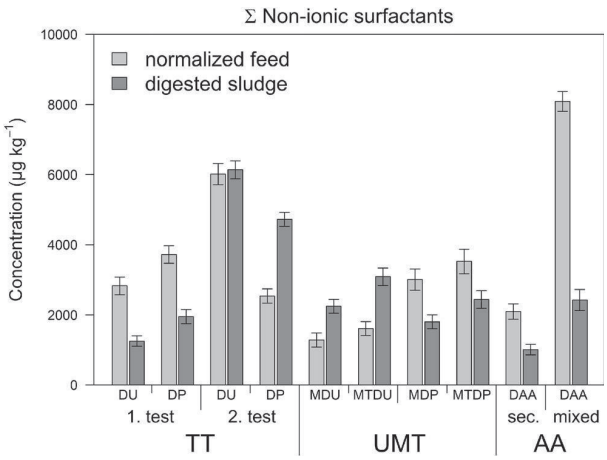


Fig. 4 Non-ionic surfactant concentration in the digested sludge samples derived from the different advanced stabilization processes. The suggested limit (50,000 µg kg^{-1}) of the EU Working Document is far above the highest value of the concentration axis

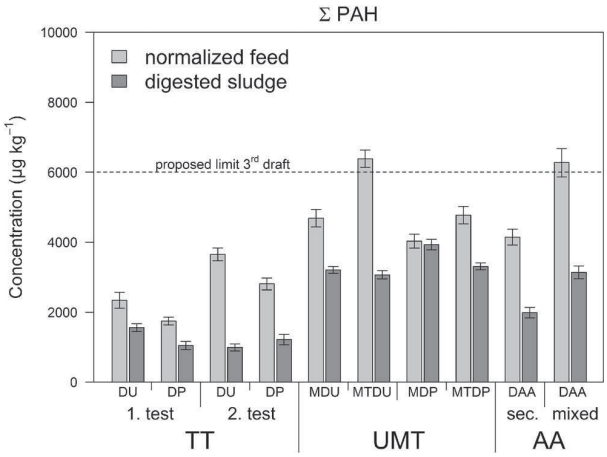


Fig. 5 PAH concentration in the digested sludge samples derived from the different advanced stabilization processes

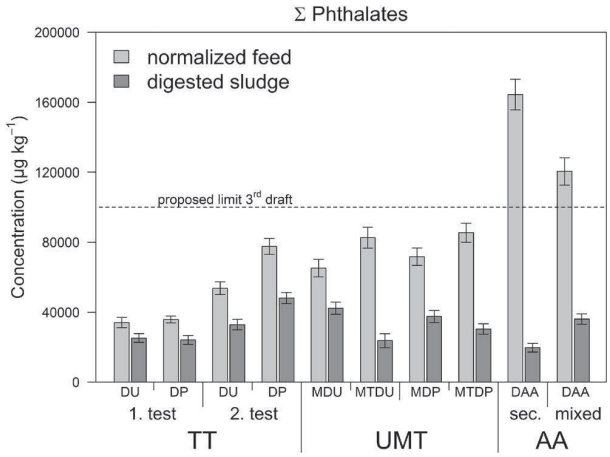


Fig. 7 Phthalate concentration in the digested sludge samples derived from the different advanced stabilization processes

Pretreating the feed sludge with thermal hydrolysis enhanced the surfactant transfer to the liquid phase, but only at high OLR (test 1) did pollutant biodegradation occur (33 %).

Finally, LAS degradation was greatly enhanced performing double-stage digestion (both UMT and AA treatments) compared to a single-stage process (TT treatment).

EOX concentrations were always far below the recommended threshold value recommended in the EU Working Document. Among the investigated treatments (Fig. 3), the UMT employing sonication gave the best removal yield (48 and 50 %), while the others between 16 and 48 %. The TT treatment resulted in lower removal yields, and the higher loading rate did not lead to an evident benefit except once (untreated) where a good removal rate (46 %) was obtained. It is worth noting that the AA treatment when operating with the mixed sludge showed a much better performance, namely, 45 % compared to 16 % (secondary sludge). The dual-stage

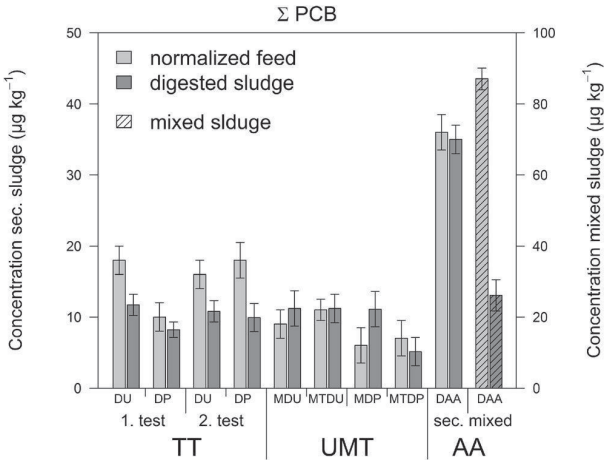


Fig. 6 PCB concentration in the digested sludge samples derived from the different advanced stabilization processes. The suggested limit (800 µg kg^{-1}) of the EU Working Document is far above the highest value of the concentration axis

mesophilic/thermophilic process integrated with ultrasound pretreatment had evident benefits for removing EOX.

Results for non-ionic surfactants showed in some cases accumulation and hence negative removal yields (Fig. 4). This quite unusual result (also observed for LAS) could be caused by a modification of the sludge structure (partial physicochemical lysis of the cells) that ultimately enhanced diffusion rate of non-ionic surfactants from the non-extractable to extractable part of the sludge (also see Patureau et al. (2008)). The TT treatment showed the highest removal yield (56 and 48 % for test 1), while the test performed at lower loading rate (test 2) showed, unexpectedly, a much worse performance. For these pollutants, pretreatment via sonication in the UMT process showed good removal yields (40 and 31 %). Also, the AA treatment when operating with the mixed sludge showed a higher performance, namely, 70 % with respect to 52 % (secondary sludge), as observed also for EOX.

PAH concentrations higher than the recommended threshold value of 6,000 µg kg^{-1} of the EU Working Document (CEC 2000) were determined only in two feed samples (Fig. 5). However, the subsequent treatment was always able to lower the residual concentration well below the threshold value. The removal yields of these pollutants showed quite different trends compared to non-ionic surfactants and EOX. Specifically, it was found that (a) for the TT treatment, test 2 (lower organic loading rate) gave higher removal yields than test 1 (73 and 57 % with respect to 33 and 40 %); (b) sonication when coupled to UMT treatment did not lead to better removal rates; and (c) AA treatment gave similar performance for secondary and mixed-digested sludge.

Results for PCBs showed that, for the TT treatment, the loading rate has practically no influence on the removal yield being the corresponding values the same within the experimental error. A similar situation can be noticed for the UMT treatment where the sonication step did not have an evident

effect (Fig. 6). The only evident result was observed for the AA treatment, where for the mixed-digested sludge, high removal yield (70 %) was obtained with respect to the negligible removal for the secondary sludge, as observed for EOX and non-ionic surfactants.

This result may be due to the poor accessibility of these pollutants, entrapped in the flocs of the secondary sludge, that impairs their biodegradability with respect to those adsorbed on surface of mixed sludge particles.

In the case of phthalates (Fig. 7), it was found that in two feed samples, the concentration was higher than the recommended threshold of 100 mg kg^{-1} (CEC 2000). However, the consequent treatment was always able to lower the residual concentration much below this threshold value. The TT treatment showed quite limited removal yields (between 26 and 39 %), while the sequential mesophilic/thermophilic UMT process gave better performances (between 35 and 71 %). The AA treatment led to the best removal rates (88 and 70 % for the secondary and mixed-digested sludge, respectively).

Fate of emerging organic micropollutants

While QACs were eliminated during the TT treatment, the thermal pretreatment did not lead to an enhanced removal of QACs in the subsequent thermophilic digestion step during both tests (Fig. 8). The total QAC removal after thermophilic digestion was slightly higher for test 1 (around 40 vs 33 %), working at higher organic loading rate. Also, for the ultrasound pretreatment (integrated in UMT process), no substantial influence on the QAC removal after combined mesophilic/thermophilic digestion was observed. In total, a substantial QAC removal (50–63 %) was observed during UMT independent on the ultrasonic pretreatment, and the majority of the total removal was obtained in mesophilic conditions (Fig. 8). QACs were efficiently removed in AA with both secondary and mixed sludges.

The highest elimination (70–90 %) of QACs obtained with the sequential anaerobic/aerobic process (AA) compared to the anaerobic processes TT (32–42 %) and UMT (50–63 %) is in accordance to literature. Tezel et al. (2006) reported no elimination of QACs under methanogenic conditions, and only slow elimination under nitrate-reducing conditions was detected (Tezel and Pavlostathis 2009). In contrast, good removal by biotransformation processes was observed in an aerobic culture (Patrauchan and Oriel 2003).

During both TT tests, except in the case of untreated feed digestion at the higher organic load (test 1), pharmaceuticals were not removed during the process (Fig. 9) and accumulated due probably to sludge structure alteration as discussed for anionic and non-ionic surfactants.

In contrast to TT, pharmaceuticals were significantly removed during the dual-stage process of the UMT treatment (Fig. 9). Analogously to the QACs, the removal occurred primarily during the mesophilic part of the subsequent treatment steps. Moreover, pretreatment via ultrasound led to no further increase in the removal of pharmaceuticals in the dual-stage digested sludge (around 57 %). When using the secondary sludge, the removal of pharmaceuticals was substantially higher (40 %) than when using the mixed sludge (no statistical significant removal).

In general, both dual-stage processes (UMT and AA) were able to remove pharmaceuticals from secondary sludge significantly. However, only UMT treatment was effective in increasing the sludge quality by reducing the actual concentration of pharmaceuticals in digested sludge compared to the feed since the removal of pharmaceuticals was lower the than the sludge reduction for both AA processes (Fig. 9 vs Online Resource 17 and 18).

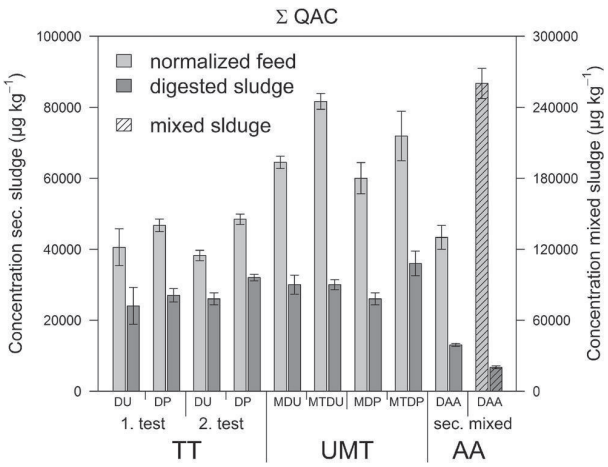


Fig. 8 QAC concentration in the digested sludge samples derived from the different advanced stabilization processes

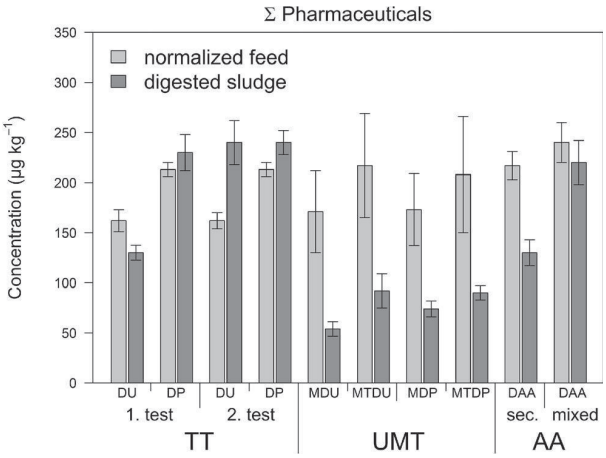


Fig. 9 Pharmaceutical concentration in the digested sludge samples derived from the different advanced stabilization processes

The TT process exhibited only little effectiveness in removing biocides and fungicides (Fig. 10). A slight removal of 25–29 % was observed during test 1. The same yield (22 %) was observed for the untreated digested sludge (TT-DU) during test 2. The apparent increase/accumulation of biocides and fungicides in TT-DP during test 2 might be a result of an underestimation of their concentrations in feed sample since the concentrations of several substances in this feed were low compared to all other feed samples. During the UMT process, pretreatment had no effect on the fate of biocides and fungicides, and removal rates were in the range of 40–48 % (Fig. 10). In accordance to QACs and pharmaceuticals, the predominant part of the total removal occurred during the mesophilic digestion. During the AA treatment, a significant load reduction for biocides and fungicides of around 50 % was observed for mixed sludge, while in the case of secondary sludge, no significant removal occurred (Fig. 10). This higher removal in mixed sludge is opposite to the observed effects for pharmaceuticals but in-line with results for QACs. Since the investigated biocides and fungicides (as well as the QACs) are in general stronger lipophilic compounds than pharmaceuticals, their enhanced removal in mixed sludge might be a result of higher sorption affinities and formation of non-extractable residues.

Ecotoxicity results

For most sludge samples, reliable EC_{50} values with reasonably tight confidence intervals could be determined in the *A. globiformis* test (Fig. 11 and Online Resource 19). For very few samples, the EC_{50} value had to be extrapolated beyond actually observed effects or tested concentrations (indicated by empty symbols in Fig. 11). The samples of secondary sludge used as feed (all "FU" samples except the second set of samples of AA where mixed sludge was used) exhibited about similar toxicity towards *A. globiformis*, while the sample of mixed sludge showed the highest toxicity of all samples with more than 50 % enzyme inhibition at the lowest tested sludge concentration (resulting in an extrapolated EC_{50} value).

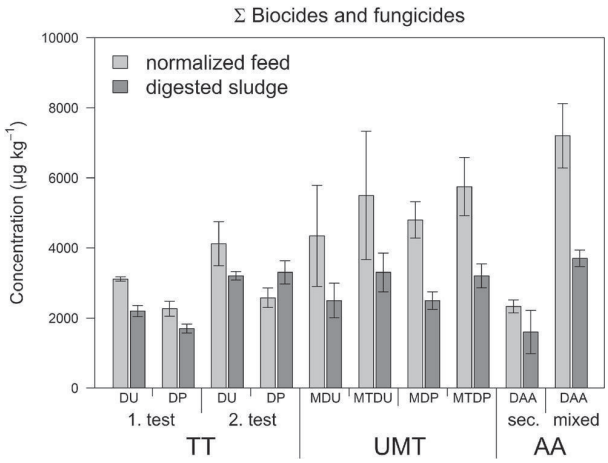


Fig. 10 Biocide and fungicide concentration in the digested sludge samples derived from the different advanced stabilization processes

For the TT process, the EC_{50} values for all samples were above the application rates in Europe and Ontario, although only in one case (TT-DP, test 1) with a safety margin of more than factor 100 related to European application rates (Fig. 11a). In both tests, the toxicity towards *A. globiformis* was hardly reduced by the pretreatment (TT-FU vs TT-FP). Yet, the thermophilic digestion step reduced toxicity only when carried out with pretreated sludge but not when conducted with untreated sludge. As a result, the digestion with pretreated sludge reduced toxicity by about factor 7 in test 1 (comparing TT-DU to TT-DP). This difference was significant based on non-overlapping confidence intervals of the EC_{50} values. Hence, the pretreatment step appeared crucial for reducing toxicity in the thermophilic digestion process. For the reduced organic loading in test 2, however, this finding was only partially confirmed as the toxicity of pretreated digested sludge was only factor 1.2 lower than that of digested sludge without pretreatment. For the samples of test 1, there was not enough material for the earthworm avoidance test. For the samples of test 2, only the feed (TT-FU) but none of the final digested sludge samples caused a limited habitat function (85 % avoidance) when amended to soil, which is in-line with the toxicity-reducing effect of this treatment process demonstrated by the *A. globiformis* test.

For the UMT process, all EC_{50} values, except one extrapolated value for the feed sludge, were above the application rates in Europe and Ontario (Fig. 11b). The sonication pretreatment apparently did not reduce toxicity as there was little difference between the FU and FP sample. The mesophilic digestion (MDU vs FU and MDP vs FP) decreased *A. globiformis* toxicity by factor 4 to 5, while the subsequent thermophilic digestion increased toxicity again. Overall, the complete treatment process achieved little reduction of toxicity, independently of the pretreatment of the secondary sludge. The EC_{50} values of the final digested sludge samples (MTDU and MTDP) provided a safety margin of about factor 100 to the usual application rate in Europe and of about factor 10 to the one in Ontario. None of the sludge samples indicated a limited habitat function (i.e., avoidance of 80 % or more) in the earthworm avoidance tests.

For the AA process, a significant reduction of toxicity towards *A. globiformis* from the feed (AA-FU) to the treated sludge (AA-DAA) was observed in both tests (Fig. 11b). The mixed sludge (feed in test 2) was more toxic than the secondary feed sludge (feed in test 2) and any other sludge tested in this study. Yet, the degree of toxicity reduction by AA was greater in the test with mixed sludge rather than in the one with secondary sludge, by factor 19 compared to factor 7,

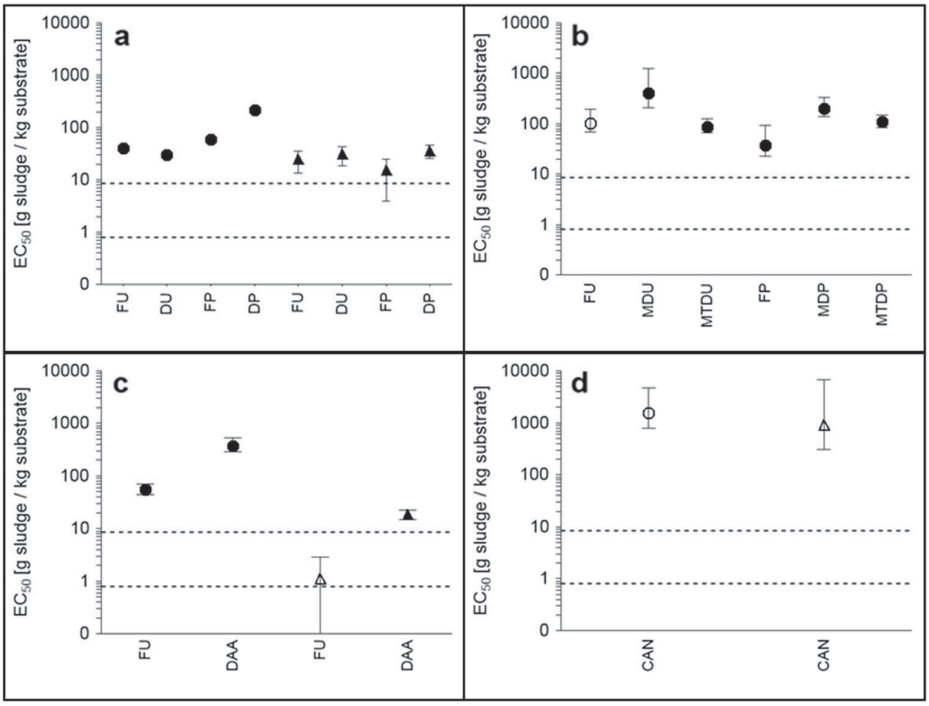


Fig. 11 Median effect concentrations (EC_{50} with 95 % confidence intervals) for the enzyme inhibition of the soil bacterium *A. globiformis* of the different sludge samples. **a** TT samples of test 1 (*circle*) and test 2 (*triangle*). **b** UMT samples. **c** AA samples with secondary (*circle*) and mixed (*triangle*) sludge. **d** Biosolid samples of reference process ("*CAN*"). *Empty symbols* indicate extrapolated EC_{50} values. *Dashed lines* at 0.8 and 8.5 g kg^{-1} refer to sludge application rates in Europe (2 t ha^{-1}) and Ontario, Canada (22 t ha^{-1}), respectively

respectively. The remaining toxicity of the final sludge reached a safety margin of more than factor 100 only for secondary but not for mixed sludge as feed in relation to usual European application rates. No earthworm avoidance behavior of sludge-amended soil was observed for the samples of the test 1, while not enough sludge was available to test the samples from test 2 for earthworm avoidance.

For the biosolid samples from Canada ("CAN"), very low and about similar toxicity was observed with safety margins to assumed application rates in Europe and Ontario of about factor 1,000 and 100, respectively. In accordance with the finding of little toxicity towards *A. globiformis*, biosolids induced no avoidance by earthworms when added to soil.

The terrestrial biotests proved suitable for measuring ecotoxicological effects of sludge and for detecting differences among different sludge samples and, hence, between the efficiency of treatment processes in reducing toxicity. The two employed test organisms belong to very different taxonomical groups and can be expected to exhibit species-specific sensitivity to contaminants and thereby react differently to the same sample. The *A. globiformis* toxicity of sludge samples is quantifiable and can be used for comparing the efficiency of various sludge treatment processes. In contrast, the earthworm avoidance test requires a rather large volume of sludge sample and could therefore only be performed at a single dosage, which did not allow quantifying the toxicity towards earthworms. Yet, the earthworm avoidance test measures the response of a key soil organism at an integrative organismal level, which allows a more straightforward extrapolation to the field. The *A. globiformis* test, on the other hand, measures the inhibition of a key enzyme that may be functional redundant in the soil microbial community.

Overall, the final digested sludge samples exhibited toxicity to the soil bacterium *A. globiformis* at concentrations that were always higher than the usual application rate of sludge to soil in Europe and the maximum allowed application rate in Ontario. With the exception of the samples produced by thermophilic digestion operated at low organic load, all samples of final digested sludge achieved safety margins of about factor 100 to the usual application rate in Europe. For earthworms, a safety margin of factor 30 was generally achieved for the final digested samples. While this are substantial safety margins for two very different organisms, it remains questionable if they can indeed be considered sufficient to account for the necessary extrapolation from the two short-term laboratory tests to a long-term field situation with organisms of other trophic levels and taxonomic groups being exposed.

The thermophilic digestion process achieved among the three processes the least toxicity reduction (at least when operated at low organic load), while the double-stage AA process appeared as the most effective process as it could greatly reduce the considerable toxicity of the mixed sludge. In comparison to the sludge samples produced by these experimental advanced treatment processes, the toxicity of the Canadian biosolids was very low in both terrestrial tests. Interestingly, a similar safety margin (about factor 100) was obtained for the biosolids with regard to the maximum allowed application rate of Ontario as for the European

sludges with regard to the European application rate. This indicates that anaerobic digestion of municipal sewage sludge, as it is done for these Canadian biosolids, may be sufficient to obtain sludge safe for agricultural application. It remains an open question, why most European sludges, even after advanced treatment, were found to exhibit much higher toxicity to *A. globiformis* than the Canadian biosolids. One explanation may be that the toxicity of sludge and biosolids is more related to the source than to the treatment, with "source" meaning the origin (and thereby contamination) of the wastewater from which the sludge was produced. The origin of the sludge within the treatment process (i.e. mixed or secondary sludge) clearly influenced toxicity as illustrated by the mixed sludge used as feed in the second AA test, as stability, too. The stability of the sludge, as measured by the VS/TS ratio, significantly correlated with the toxicity to *A. globiformis* in the here investigated 18 samples (Table 10): The more stable the sludge, the lower the toxicity was. This may indicate that ammonium released from the less stabilized sludge caused the toxicity in *A. globiformis*. Römbke et al. (2009) reported no correlation between the toxicity of incineration ashes to *A. globiformis* and their concentrations of heavy metals. Römbke et al. (2009) detected a clear and significant correlation between toxicity to *A. globiformis* and ammonium-nitrate concentrations (Spearman rank *R* of 0.68), which supports the explanation of ammonia release as reason for the greater toxicity of less stabilized sludge. None of the sum concentrations of the various pollutant classes exhibited significant correlations with toxicity to *A. globiformis* (Table 10), which is not really surprising as these groups likely contain substances with very different modes of action towards the tested bacterium. The concentrations of only three of the measured individual pollutants (carbamazepine, triclocarban and napthalene) exhibited significant correlations with toxicity to *A. globiformis*, while for all other pollutants that were detected in all of the sludge samples, the correlations were non-significant.

Table 10 Spearman rank correlation coefficients between toxicity to *A. globiformis* (EC_{50}) and characteristics of sludge samples

	R values
Stability index VS/TS	**−0.61**
Soluble COD	−0.16
Soluble N-NH_4	0.32
Sum of PAHs	0.13
Sum of PCBs	−0.13
Sum of phthalates	−0.38
Sum of QACs	−0.02
Sum of pharmaceuticals	−0.34
Sum of biocides and fungicides	−0.31
EOX	−0.20
Carbamazepine	**−0.56**
Triclocarban	**−0.57**
Naphthalene	**−0.54**

Spearman's correlation coefficients for pollutants are shown for summed concentrations and for individual pollutants (detected in all samples) with significant correlations. Canadian biosolids not included (n=18). Significant correlations (p<0.05) are indicated in bold

There is no evidence for toxicity of carbamazepine to bacteria (Jos et al. 2003) and no reports for naphthalene toxicity in *A. globiformis*, while for triclocarban, a concentration-dependent toxicity to *A. globiformis* can be explained by the intended antibacterial effects of this microbiocide. However, for the closely related microbiocide triclosan, no correlation was detected, which remains unexplained.

Conclusions

1. The stability index of the digested sludge, evaluated by means of VS/TS ratio, was neither affected by the pretreatment nor by the organic load variation. The thermophilic anaerobic digestion, because of the efficient particulate degradation, worsened the quality of the supernatant (in terms of ammonia and COD) and sludge dewaterability, either alone (as in TT) or coupled with a mesophilic stage (as in UMT). On the contrary, the integration of an aerobic posttreatment improved the stability of the digested sludge and the quality of the supernatant due to significant ammonia and COD removal.
2. The concentrations of heavy metals in the raw sludges remained almost constant during the entire experimentation, with Zn the most abundant metal (440–764 mg kg^{-1}), while Cd was present at the lowest concentration (0.8–1.2 mg kg^{-1}). No removal occurred during the investigated processes, but because of the mass loss during the treatments, the effective heavy metal concentration increased. Nevertheless, in all cases, the final heavy metal concentrations were lower than the EU limits, allowing the application of these sludges to agricultural land.
3. The concentrations of the conventional organic pollutants (EOX, LAS, NPEs, PCBs, PAHs, and phthalates) in the feed just in few cases exceed the recommended thresholds set in the EU Working Document (CEC 2000). However, the subsequent stabilization treatment was always able to lower the residual concentration well below such a threshold value. The enhanced stabilization processes did not give the same results for all investigated conventional organic pollutants. However, in many cases, it was found that the dual-stage mesophilic/thermophilic process integrated with ultrasound pretreatment has evident benefits for removing the investigated organics.

4. The concentrations of emerging organic pollutants in the feed sludge were lower compared to available literature data. None of the pretreatments (thermal hydrolysis or ultrasound) influenced the removal efficiencies for the emerging organic pollutants. QACs and biocides/fungicides were removed at higher rates than the investigated pharmaceuticals. Despite the weight losses during the treatments, a substantial increase in pollutant concentration in the final sludge compared to the increase was only observed for the TT process. Therefore, AA and UMT processes are regarded as more efficient in reducing emerging organic pollutants than the TT process.
5. The best reduction of *A. globiformis* toxicity was achieved by the sequential process anaerobic/aerobic, followed by the dual-stage mesophilic/thermophilic and then the thermophilic digestion, alone or integrated within thermal hydrolysis. The determined toxicity of the final digested sludge provided a safety margin about factor 100 to the usual European application rates. While this appears as a considerably large margin, it has to be taken into account that in case of only few tested organisms, a safety margin of 1,000 may rather be warranted. The toxicity to *A. globiformis* appears to primarily relate not only to incomplete stabilization of sludge but also to some conventional (naphthalene) and emerging organic (triclocarban and carbamazepine) pollutants.

Overall, it can be concluded that sequential double-stage digestion, whether fully anaerobic mesophilic/thermophilic or anaerobic/aerobic, affected positively the quality of the digested sludges, compared to single-stage digestion. On the contrary, pretreatments as ultrasounds or thermal hydrolysis did not influence significantly the quality of the digested sludges.

Toxicity reduction during the enhanced stabilization processes (in particular during anaerobic/aerobic digestion) was strictly related to efficient removal of organic micropollutants, both conventional and emerging ones.

Acknowledgments This work was supported by the EU ROUTES project funded from the European Union's Seventh Programme for research, technological development, and demonstration under grant agreement no. 265156. Giuseppe Bagnuolo, Ruggero Ciannarella and Vito Locaputo (IRSA-CNR, Bari, Italy) are acknowledged for carrying out determinations of conventional organic micropollutants. Björn Jacobs and Lennart Lörke (BfG, Koblenz, Germany) are thanked for their help during the analysis of emerging organic pollutants.

References

Abad E, Martínez K, Planas C, Palacios O, Caixach J, Rivera J (2005) Priority organic pollutant assessment of sludges for agricultural purposes. Chemosphere 61(9):1358–69

Aparicio I, Santos JL, Alonso E (2009) Limitation of the concentration of organic pollutants in sewage sludge for agricultural purposes: a case study in South Spain. Waste Manage 29:1747–1753

APHA Standard Methods for the Examination of Water and Wastewater (1998) 20th edition, American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA

Barret M, Cea-Barcia C, Guillon A, Cerrére H, Patureau D (2010) Influence of feed characteristics on the removal of micropollutants during the anaerobic digestion of contaminated sludge. J Hazard Mater 181:241–247

Barron J, Tobin J, Paull B (2008) Multi-residue determination of pharmaceuticals in sludge and sludge enriched soils using pressurized liquid extraction, solid phase extraction and liquid chromatography with tandem mass spectrometry. J Environ Monit 10:353

Berset JD, Holzer R (1996) Determination of coplanar and ortho substituted PCBs in some sewage sludges of Switzerland using HRGC/ECD and HRGC/MSD. Chemosphere 32(12): 2317–2333

Bertanza G., Canato M, Laera G, Tomei M C (2014). Method for technical, economic and environmental assessment of advanced sludge processing routes. Environmental Science and Pollution Research (in press)

Blanchard M, Teil MJ, Ollivon D, Legenti L, Chevreuil M (2004) Polycyclic aromatic hydrocarbons and polychlorobiphenyls in wastewaters and sewage sludges from the Paris area (France). Environ Res 95(2):184–197

Carballa M, Omil F, Lema JM, Llompart M, Garcia-Jares C, Rodriguez I, Gomez M, Ternes T (2004) Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. Water Res 38(12):2918–2926

Carballa M, Omil F, Ternes T, Lema JM (2007) Fate of pharmaceutical and personal care products (PPCPs) during anaerobic digestion of sewage sludge. Water Res 41(10):2139–2150. doi:10.1016/j.watres.2007.02.012

Carballa M, Omil F, Lema J (2009) Influence of different pretreatments on anaerobically digested sludge characteristics: suitability for final disposal. Water, Air, Soil Pollut 199(1–4):311–321. doi:10.1007/s11270-008-9880-z

CEC (1986) Council Directive of 12 june 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture. Council of the European Communities (Directive 86/278/EEC).

CEC (2000) Council Directive of 27 April 2000 on Working Document on Sludge—third draft. Brussels: European Commission DG Environment. Retrieved from http://ec.europa.eu/environment/waste/sludge/pdf/sludge_en.pdf

CEN (2005) Characterization of waste—preparation of waste samples for ecotoxicity tests. EN 14735, Brussels, Belgium

Clara M, Scharf S, Scheffknecht C, Gans O (2007) Occurrence of selected surfactants in untreated and treated sewage. Water Res 41(19):4339–4348

Clarke BO, Smith SR (2011) Review of 'emerging' organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids. Environ Int 37:226–247

Cogger CG, Forge TA, Neilsen GH (2006) Biosolids recycling: nitrogen management and soil ecology. Can J Soil Sci 86:613–620

Díaz-Cruz MS, García-Galán MJ, Guerra P, Jelic A, Postigo C, Eljarrat E, Farré M, Barceló D (2009) Analysis of selected emerging contaminants in sewage sludge. TrAC 28:1263–1275

Dionisi D, Bertin L, Bornoroni L, Capodicasa S, Papini MP, Fava F (2006) Removal of organic xenobiotics in activated sludges under aerobic conditions and anaerobic digestion of the adsorbed species. J Chem Technol Biotechnol 81:1496–1505

EC-European Commission (2010) Disposal and recycling routes for sewage sludge. Part 2—Regulatory report, European Commission,

DG Environment. Office for Official Publications of the European Communities, Luxembourg

El-Hadj BT (2006) Biodegradation of organic micropollutants in thermophilic and mesophilic anerobic digestion of sewage sludge. Dissertation, Universitat de Barcelona

El-Hadj BT, Dosta J, Mata-Alvarez J (2006) Biodegradation of PAH and DEHP micro-pollutants in mesophilic and thermophilic anaerobic sewage sludge digestion. Water Sci Technol 53(8):99–107

EPA 2006 Biosolids technology fact sheet—multi-stage anaerobic digestion, US Environmental Protection Agency Washington DC, Technical report EPA832/F/06/031

Erhardt W and Prüß A (2001) Organic contaminants in sewage sludge for agricultural use. Report for the European Commission Joint Research Centre, Institute for Environment and Sustainability Soil and Waste Unit

Eriksen GS, Amundsen CE, Bernhoft A, Eggen T, Grave K, Halling-Sørensen B, et al. (2009) Risk assessment of contaminants in sewage sludge applied on Norwegian soils. Norway: Panel on Contaminants in the Norwegian Scientific Committee for Food Safety

Finney DJ (1978) Statistical methods in biological assay, 3rd edn. Charles Griffin & Company Ltd., London

Gallipoli A, Gianico A, Gagliano MC, Braguglia CM (2014) Potential of high-frequency ultrasounds to improve sludge anaerobic conversion and surfactants removal at different food/inoculum ratio. Biores Technol, in press doi: 10.1016/j.biortech.2014.02.084 .

Gianico A, Braguglia CM, Mascolo G, Mininni G (2013a) Partitioning of nutrients and micropollutants along the sludge treatment line: a case study. Environ Sci Pollut Res 20(9):6256–65. doi:10.1007/s11356-013-1686-x

Gianico A, Braguglia CM, Cesarini R, Mininni G (2013b) Reduced temperature hydrolysis at 134 °C before thermophilic anaerobic digestion of waste activated sludge at increasing organic load. Biores Technol 143:96–103

Gianico A, Braguglia CM, Gallipoli A, Mininni G (2014) Innovative two-stage mesophilic/thermophilic anaerobic degradation of sonicated sludge: performances and energy balance. Environ Sci Pollut Res. doi:10.1007/s11356-014-3123-1

Hijaz M (2007) The fate of organic chemicals in wastewater and sewage sludge. MSc thesis, http://randd.defra.gov.uk/Document.aspx?Document=SP0547_7266_FRA.pdf

Ismail ZZ, Tezel U, Pavlostathis, SG (2010) Sorption of Quaternary Ammonium Compounds to Municipal Sludge. Water Research 44(7):2303–2313

ISO (2008a) Soil quality—guidance on the assessment of tests applied in the field of ecotoxicological characterization of soils and soil materials. ISO 17616. Geneve, Switzerland

ISO (2008b) Soil quality—avoidance test for evaluating the quality of soils and the toxicity of chemicals. Test with Earthworms (Eisenia fetida/andrei). ISO 17512-1. Geneve, Switzerland

ISO (2012) Soil quality—quality of solid samples—solid contact test using the dehydrogenase activity of Arthrobacter globiformis. Draft guideline ISO 18187. Geneve, Switzerlan

Jahnke A, Gandrass J, Ruckb W (2004) Simultaneous determination of alkylphenol ethoxylates and their biotransformation products by liquid chromatography/electrospray ionisation tandem mass spectrometry. J Chromatogr A 1035:115–122

Jones KC, Northcott GL (2000) Organic contaminants in sewage sludges: a survey of UK samples and a consideration of their significance. Final Report to the Department of the Environment, Transport and the Regions, Water Quality Division

Jos A, Repetto G, Rios JC, Hazen MJ, Molero ML, del Peso A, Salguero M, Fernandez-Freire P, Perez-Martin JM, Camean A (2003) Ecotoxicological evaluation of carbamazepine using six different model systems with eighteen endpoints. Toxicol In Vitro 17:525–532

Kreuzinger N, Fuerhacker M, Scharf S, Uhl M, Gans O, Grillitsch B (2007) Methodological approach towards the environmental significance of uncharacterized substances-quaternary ammonium compounds as an example. Desalination 215(1–3):209–222

Langenbach T, Pfeifer W, Freire LR, Sarpa M, Paim S (1994) Heavy metals in sludge from the sewage treatment plant of Rio de Janeiro. Environm Technol 5:997–1000

Levantesi C, Beimfohr C, Blanch AR, Lucena F, Tomei MC, Gianico A, Mininni G, Carducci A (2014) Sludge hygienization by innovative sludge treatment processes to assure safe land spreading. Environ Sci Pollut Res: submitted to this special issue

Lukicheva I, Pagilla K, Rohloff G, Kunetz T(2009) To do Class A or Not? What to do to enhance sludge processing?. Water Environment Federation, WEFTEC: 1256-1273

Madsen T, Kristensen P (1997) Effects of bacterial inoculation and nonionic surfactants on degradation of polycyclic aromatic hydrocarbons in soil. Environ Toxicol Chem 16:631–637

Martinez-Carballo E, Gonzalez-Barreiro C, Sitka A, Kreuzinger N, Scharf S, Gans O (2007) Determination of selected quaternary ammonium compounds by liquid chromatography with mass spectrometry. Part Ii. Application to sediment and sludge samples in Austria. Environ Pollut 146(2):543–547

Mascolo G, Balest L, Cassano D, Laera G, Lopez A, Pollice A, Salerno C (2010a) Biodegradability of pharmaceutical industrial wastewater and formationof recalcitrant organic compounds during aerobic biological treatment. Biores Technol 101(8):2585–2591

Mascolo G, Locaputo V, Mininni G (2010b) New perspective on the determination of flame retardants in sewage sludge by using ultra high pressure liquid chromatography-tandem mass spectrometry with different ion sources. J Chromatogr A 1217(27):4601–4611

Metcalf L, Eddy HP (2003) Wastewater engineering: treatment and reuse, 4th edn. McGraw-Hill, New York

Moser H, Roembke J (2009) The ecotoxicological characterisation of waste—results and experiences from a European ring test. Springer Ltd., New York, p 308

Novak JT, Sadler MS, Murthy SN (2003) Mechanism of floc destruction during anaerobic and aerobic digestion and the effect on conditioning and dewatering of biosolids. Water Res 37:3136–3144

NRC-National Research Council (2002) Biosolids applied to land: advancing standards and practices (Prepublication Copy). National Academy Press, Washington, D.C

OECD (1984): Test No. 207: earthworm, acute toxicity tests. OECD Guidelines for the Testing of Chemicals. Paris, France

ONTARIO REGULATION-OR (2009) 338/09 made under the NUTRIENT MANAGEMENT ACT, 2002, amended 2009 and amending O.Reg. 267/03. The Ontario Gazette:http://www.elaws.gov.on.ca/html/source/regs/english/2009/elaws_src_regs_r09338_e.htm

Parravicini V, Svardal K, Hornek R, Kroiss H (2008) Aeration of anaerobically digested sewage sludge for COD and nitrogen removal: optimization at large-scale. Water Sci Technol 57:257–264

Patrauchan MA, Oriel PJ (2003) Degradation of benzyldimethylalkylammonium chloride by *Aeromonas hydrophila* sp. K J Appl Microbiol 94(2):266–272

Patureau D, Trably E (2006) Impact of anaerobic and aerobic processes on polychlorobiphenyl removal in contaminated sewage sludge. Biodegradation 17:9–17

Patureau D, Delgenes N, Delgenes JP (2008) Impact of sewage sludge treatment processes on the removal of the endocrine disrupters nonylphenol ethoxylates. Chemosphere 72:586–591

Paulsrud B, Wien A, Nedland KT (1998) A survey of toxic organics in Norwegian sewage sludge, compost and manure. Paper presented at 8th International Conference of the FAO. Phys Eng Sci 367:4005–4041

Radjenovic J, Jelic A, Petrovic M, Barcelo D (2009) Determination of pharmaceuticals in sewage sludge by pressurized liquid extraction

(PLE) coupled to liquid chromatography-tandem mass spectrometry (LC-MS/MS). Anal Bioanal Chem 393:1685–1695

Riau V, De la Rubia MA, Pérez M (2010) Temperature-phased anaerobic digestion (TPAD) to obtain class A biosolids: a semicontinuous study. Biores Technol 101:2706–2712

Römbke J, Moser TH, Moser H (2009) Ecotoxicological characterization of 12 incineration ashes (MWI) using 6 labor-atory tests. Waste Manage 29:2475–2482

Sastre J, Sahuquillo A, Vidal M, Rauret G (2002) Determination of Cd, Cu, Pb and Zn in environmental samples: microwave-assisted total digestion versus aqua regia and nitric acid extraction. Anal Chim Acta 462:59–72

Smith SR (2009) Organic contaminants in sewage sludge (biosolids) and their significance for agricultural recycling. Philos Trans R Soc A-Math Phys Eng Sci 367:4005–4041

Smith SR and Riddell-Black D (2007) Sources and impacts of past, current and future contamination of soil. Appendix 2: Organic contaminants SP0547. Defra Science Directorate. London, UK: Department for Environment Food and Rural Affairs

Spongberg AL, Witter JD (2008) Pharmaceutical compounds in the wastewater process stream in Northwest Ohio. Sci Total Environ 39:148–157

Taylor M, Elliott HA (2013) Influence of water treatment residuals on dewaterability of wastewater biosolids. Water Sci and Technol 67(1):180–6. doi:10.2166/wst.2012.553

Tchobanoglous G, Burton FL (1996) Wastewater engineering treatment disposal and reuse. McGraw-Hill International Edition, Singapore, pp 84–827

Tezel U, Pavlostathis SG (2009) Transformation of benzalkonium chloride under nitrate reducing conditions. Environ Sci Technol 43(5): 1342–1348

Tezel U, Pierson JA, Pavlostathis SG (2006) Fate and effect of quaternary ammonium compounds on a mixed methanogenic culture. Water Res 40(19):3660–3668

Thornton I, Butler D, Docx P, Hession M, Makropoulos C, McMullen M, Nieuwenhuijsen M, Pitman A, Rautiu R, Sawyer R, Smith S,White D, Wilderer P, Paris S, Marani D, Braguglia C, Palerm J (2001) Pollutants in urban waste water and sewage sludge. Final report prepared for European Commission Directorate- General Environment

Tomei MC, Carozza N (2014) Sequential anaerobic/aerobic process for enhanced sludge stabilization: comparison of the digestion performance for mixed and waste activated sludge. Environ Sci Pollut Res: submitted to this special issue

Tomei MC, Rita S, Mininni G (2011) Performance of sequential anaerobic/aerobic digestion applied to municipal sewage sludge. J Environ Manage 92:1867–1873

Trably E, Patureau D, Delgene's JP (2003) Enhancement of polycyclic aromatic hydrocarbons (PAHs) removal during anaerobic treatment of urban sludge. Wat Sci Technol 48:53–60

USEPA-United States Environmental Protection Agency (1987) Dewatering municipal wastewater sludge. EPA/625/1–87/ 014. Office of Research and Development, Washington, DC

Vesilind PA (1988) Capillary suction time as a fundamental measure of sludge dewaterability. J WPCF 60(2):215–219

Wang LK, Shammas NK, Hung Y-T (2009) Engineering and management of agricultural land application in biosolids engineering and management. Humana Press, Totowa, NJ

Wick A, Fink G, Ternes TA (2010) Comparison of electrospray ionization and atmospheric pressure chemical ionization for multi-residue analysis of biocides, UV-filters and benzothiazoles in aqueous matrices and activated sludge by liquid chromatography-tandem mass spectrometry. J Chromatogr A 1217(14):2088–2103

Wilson CA, Novak JT (2009) Hydrolysis of macromolecular components of primary and secondary wastewater sludge by thermal hydrolytic pretreatment. Water Res 43:4489–4498

Yukseler H, Tosun I, Yetis U (2007) A new approach in assessing slurry filterability. J Memb Sci 303:72–79

Environ Sci Pollut Res (2015) 22:7236
DOI 10.1007/s11356-015-4127-1

ERRATUM

Erratum to: Quality assessment of digested sludges produced by advanced stabilization processes

C. M. Braguglia · A. Coors · A. Gallipoli · A. Gianico · E. Guillon · U. Kunkel · G. Mascolo · E. Richter · T. A. Ternes · M. C. Tomei · G. Mininni

Published online: 7 February 2015

Erratum to: Environ Sci Pollut Res
DOI 10.1007/s11356-014-3090-6

The 2nd affiliation of Elisabeth Richter is missing.
She is also affiliated to: Goethe-University Frankfurt/Main, Department Aquatic Ecotoxicology, Frankfurt/Main, Germany

The online version of the original article can be found at http://dx.doi.org/10.1007/s11356-014-3090-6.

C. M. Braguglia (✉) · A. Gallipoli · A. Gianico · M. C. Tomei · G. Mininni
Istituto di Ricerca sulle Acque-CNR, Area della Ricerca RM1, Monterotondo, Rome, Italy
e-mail: braguglia@irsa.cnr.it

A. Coors · E. Richter
ECT Oekotoxikologie, Flörsheim/Main, Germany

E. Guillon
ICMR UMR CNRS 7312, Université de Reims Champagne-Ardenne, Reims, France

U. Kunkel · T. A. Ternes
Federal Institute of Hydrology (BfG), Koblenz, Germany

G. Mascolo
Istituto di Ricerca sulle Acque-CNR, Bari, Italy

E. Richter
Goethe-University Frankfurt/Main, Department Aquatic Ecotoxicology, Frankfurt/Main, Germany

Quality assessment of digested sludge produced by different advanced stabilization processes

C.M. Braguglia*[1], A. Coors[2], A. Gallipoli[1], A. Gianico[1], E. Guillon[3], U. Kunkel[4], G. Mascolo[5],E. Richter[2], M.C. Tomei[1], T.A. Ternes[4], G. Mininni[1]

[1]*Istituto di Ricerca sulle Acque-CNR, Area della Ricerca RM1, Monterotondo (Rome), Italy*
[2]*ECT Oekotoxikologie, Flörsheim/Main, Germany*
[3]*GRECI (Groupe de Recherche En Chimie Inorganique), Universite´de Reims Champagne-Ardenne, Reims, France*
[4]*Federal Institute of Hydrology (BfG), Koblenz, Germany*
[5]*Istituto di Ricerca sulle Acque-CNR, Bari, Italy*

*Corresponding author. Tel.: +39 0690672798; fax: +39 0690672787.
E-mail address: braguglia@irsa.cnr.it

Online Resource 1. List of analysed linearalkylbenzenesulphonates (LAS) by HPLC/MS

Compound	Target ion (m z^{-1})
C10-LAS	297
C11-LAS	311
C12-LAS	325
C13-LAS	339
C14-LAS	353
C8-LAS (intern std)	269

Online Resource 2. List of analysed nonylphenolethoxylates by UPLC/MS-MS.

Compound	abbreviation	CAS #	LOQ (µg kg^{-1})	polarity	Precursor ion (m z^{-1})	Product ion (m z^{-1})
4-tert-Octylphenol monoethoxylate	OP1EO	2315-67-5	1.9	(+)	268	113
4-tert-Octylphenol diethoxylate	OP2EO	2315-61-9	0.8	(+)	312	183
onylphenol monoethoxylate (technical)	NP1EO	n.a.	0.8	(+)	282	127
Nonylphenol diethoxylate (technical)	NP2EO	n.a.	0.8	(+)	326	183
$^{13}C_6$ 4-tert-Octylphenol diethoxylate, labeled	OP2EO-C13			(+)	318	189
4-n-octylphenol	4n-OP	1806-26-4	0.8	(-)	205	106
4-t-octylphenol	t-OP	140-66-9	3.7	(-)	205	133
4-t-nonylphenol (technical)	t-NP	25154-52-3	0.8	(-)	219	133
4-nonylphenol	4n-NP	104-40-5	0.8	(-)	219	106
$^{13}C_6$ 4-t-octylphenol, labeled	t-OP-C13			(-)	211	139

Online Resource 3. List of analysed PAHs by GC/MS.

Compound	CAS #	LOQ (µg kg^{-1})	molecular ion (m z^{-1})
Naphthalene	91-20-3	5	128
Acenaphthylene	208-96-8	5	152
Acenaphthene	83-32-9	5	154
Fluorene	86-73-7	5	166
Phenanthrene	85-01-8	5	178
Anthracene	120-12-7	5	178
Fluoranthene	206-44-0	5	202
Pyrene	129-00-0	5	202
Benzo[a]anthracene	56-55-3	5	228
Chrysene	218-01-9	5	228
Benzo[b]fluoranthene	205-99-2	5	252
Benzo[k]fluoranthene	207-08-9	5	252
Benzo[a]pyrene	50-32-8	5	252
Indeno[1,2,3-cd]pyrene	193-39-5	12.5	276
Dibenzo[a,h]anthracene	53-70-3	12.5	278
Benzo[g,h,i]perylene	191-24-2	12.5	276

Online Resource 4: List of analysed PCBs by GC/MS-MS.

Compound	CAS #	LOQ (µg kg^{-1})	Precursor ion (m z^{-1})	Product ions scan range (m z^{-1})
PCB-28	7012-37-5	0.5	258	160-210
PCB-52	35693-99-3	0.5	292	230-270
PCB-101	37680-73-2	0.5	326	240-310
PCB-118	31508-00-6	0.5	326	240-310
PCB-138	35065-28-2	0.5	360	270-340
PCB-153	35065-27-1	0.5	360	270-340
PCB-180	35065-29-3	0.5	396	310-370
$^{13}C_{12}$PCB-104, labelled	234432-89-4		338	260-320

Online Resource 5. List of analysed phthalates by GC/MS.

Compound	CAS #	LOQ (µg kg^{-1})	molecular ion (m z^{-1})
Dimethyl phthalate	131-11-3	5	194
Diethyl phthalate	84-66-2	5	222
Di-n-butyl phathalate	84-74-2	5	278
Butyl benzyl phathalate	85-68-7	5	312
Bis (2-ethylexyl) phthalate	117-81-7	5	390
Di-n-octyl phathalate	117-84-0	50	390

Online Resource 6. List of analyzed quaternary ammonia compounds (QACs)

Compound	CAS # (*)	LOQ (µg kg^{-1})	polarity	Precursor ion (m z^{-1})	Product ions (m z^{-1})
Benzethonium chloride	112-00-5	200	(+)	412	91/72
Benzyldimethyldodecylammonium chloride	1119-97-7	150	(+)	304	91/212
Benzyldimethylhexadecylammonium chloride	57-09-0	300	(+)	360	91/268
Benzyldimethyloctadecylammonium chloride	2390-68-3	150	(+)	388	91/296
Benzyldimethyltetradecylammonium chloride	124-03-8	150	(+)	332	91/240
Benzyltributylammonium chloride	7281-04-1	50	(+)	276	142/187
Bis(triphenylphos-phoranylidene)	21050-13-5	100	(+)	538	306/384
Dibenzyldimethylammonium chloride	197961-03-8	100	(+)	266	91/134
Didecyldimethylammonium chloride	121-54-0	300	(+)	326	186/43
Didodecyldimethylammonium chloride	21050-13-5	100	(+)	382	214/43
Ditetradecyldimethylammonium chloride	3282-73-3	250	(+)	438	242/57
Dodecyltrimethylammonium chloride	206752-43-4	350	(+)	228	57/60
Ethylhexadecyldimethylammonium chloride	68105-02-2	350	(+)	299	74/43
Hexadecyltrimethylammonium chloride	37026-88-3	150	(+)	284	60/57
Octadecyltrimethylammonium chloride	1643-19-2	300	(+)	312	60/43
Tetrabutylammonium chloride	25316-59-0	250	(+)	242	142/100
Tetradecyltrimethylammonium chloride	26616-35-3	350	(+)	256	60/43
Tetrapropylammonium chloride	197961-03-8	100	(+)	186	114/144
Tributylmethylammonium chloride	1941-30-6	150	(+)	200	144/100
Vinylbenzyltrimethylammonium chloride	1812-53-9	100	(+)	176	117/58

(*) CAS number for the chloride salt, some substances was alternatively purchased as bromide salt

Online resource 7. List of analyzed pharmaceuticals, biocides, and fungicides

Compound	Compound class	CAS #	LOQ (µg kg^{-1})	polarity	Precursor ion (m z^{-1})	Product ion (m/z)
Acetylsulfamethoxazole (Ac-SMX)	Pharmaceutical	21312-10-7	15	(+)	296	136/188
Bezafibrate	Pharmaceutical	41859-67-0	2.5	(-)	360	274/154
Carbamazepine	Pharmaceutical	298-46-4	2.5	(+)	237	194/179
Clarithromycin	Pharmaceutical	81103-11-9	20	(+)	748.4	158/590
Dihydrodihydroxycarbamazepine (DHDH-CBZ)	Pharmaceutical	35079-97-1	25	(+)	271	236/180
Dihydrohydroxycarbamazepine (DHH-CBZ)	Pharmaceutical	29331-92-8	25	(+)	255	237/194
Diclofenac	Pharmaceutical	15307-86-5	5.0	(+)	296	250/215
Metoprolol	Pharmaceutical	51384-51-1	25	(+)	268	116/74
Primidone	Pharmaceutical	125-33-7	7.5	(+)	219	190/162
Sulfamethoxazole (SMX)	Pharmaceutical	723-46-6	5.0	(+)	254	156/188
Trimethoprim	Pharmaceutical	738-70-5	2.5	(+)	291	230/261
Venlafaxine	Pharmaceutical	93413-69-5	7.5	(+)	278	260/58
2-Benzyl-4-chlorophenol	Biocide	120-32-1	50	(-)	217	35/181
Carbendazim	Fungicide	10605-21-7	5.0	(+)	192	160/132
Climbazole	Fungicide	38083-17-9	5.0	(+)	293	197/69
Diuron	Biocide	330-54-1	6.5	(+)	233/235	72/72
Fluconazole	Fungicide	86386-73-4	6.5	(+)	307	220/238
Irgarol (Cybutryn)	Biocide	28159-98-0	6.5	(+)	254	198/83
Isoproturone	Biocide	4123-59-6	5.0	(+)	207	72/165
Propiconazole	Fungicide	60207-90-1	25	(+)	342/344	159/161
Tebuconazole	Fungicide	107534-96-3	2.5	(+)	308/310	70/70
Terbutryn	Biocide	886-50-0	5.0	(+)	242	186/91
Mecoprop	Biocide	93-65-2	25	(-)	213	141/71
Triclocarban (TCC)	Biocide	101-20-2	3.0	(-)	313/315	160/162
Triclosan (TCS)	Biocide	3380-34-5	12.5	(-)	287/289	35/37

Online Resource 8. EU limit values for concentration of heavy metals in biosolids for use on land

Element	Limit Values (mg kg^{-1})	
	Directive 86/279/EEC	3rd Draft (CEC 2000)
Cd	20-40	10
Cr	-	1000
Cu	1000-1750	1000
Hg	16-25	10
Ni	300-400	300
Pb	750-1200	750
Zn	2500-4000	2500

Online Resource 9. Concentration (mg kg $^{-1}$) of heavy metals in the feed sludges of TT

	Test 1		**Test 2**	
	TT-FU	TT-FP	TT-FU	TT-FP
Cd	0.8±0.04	0.7±0.03	1.2±0.04	1±0.03
Cr	28.6±0.3	26.4±0.2	25.2±0.1	25.7±0.2
Cu	205±1	192±1	241±1.5	204±1.2
Ni	30±0.5	22±0.2	29±0.5	38.7±0.3
Pb	94.3±0.5	88.5±0.4	93.4±0.4	87±0.4
Zn	662±3	651±3	764±4	616±3
Hg	n.d.	n.d.	n.d.	n.d.

n.d. not determined

Online Resource 10. Concentration (mg kg $^{-1}$) of heavy metals in the feed sludges of UMT

	UMT-FU	UMT-FP
Cd	1.2±0.05	0.8±0.03
Cr	19.6±0.1	19.7±0.1
Cu	144±1	137±1
Ni	14±0.1	13.5±0.1
Pb	58±0.3	57±0.2
Zn	442±2	423±2
Hg	0.5±0.04	0.5±0.04

Online Resource 11. Concentration (mg kg $^{-1}$) of heavy metals in the feed sludges of AA

	AA-FU (secondary)	AA-FU (mixed)
Cd	1±0.03	1±0.03
Cr	26.1±0.3	26±0.2

Cu	234±1.2	232±1.5
Ni	38.1±0.2	28±0.1
Pb	94±0.5	116±0.4
Zn	720±4	771±4
Hg	n.d.	n.d.

n.d. not determined

Online Resource 12. Proposed limit concentrations for organic pollutants in biosolids (CEC 2000)

Pollutants	**Limit Concentration (mg kg^{-1})**
Halogenated organic compounds	500
Linear alkylbenzene sulphonates	2600
Di(2-ethylhexyl)phthalate	100
Nonylphenol and nonylphenolethoxylates	50
PAHs	6
Polychlorinated biphenyls	0.8
Polychlorinated dibenzodioxins/dibenzofurans	0.0001 (total equivalents)

Online Resource 13. Concentration of conventional organic micropollutants in the feed sludges of TT

		Test 1	
		TT-FU	TT-FP
EOX	µgCl kg^{-1}	4650±100	5660
total non-ionic surfactants	µg kg^{-1}	2122±48	2799±66
total anionic surfactants	mg kg^{-1}	206±10	310±15
total PAHs	µg kg^{-1}	1733±167	1277±83
total PCBs	µg kg^{-1}	12.6±0.7	7.3±0.4
total Phthalates	µg kg^{-1}	25000±2167	25793±1286
		Test 2	
		TT-FU	TT-FP
EOX	µgCl kg^{-1}	8370±240	7710±220
total non-ionic surfactants	µg kg^{-1}	3991±95	1581±48
total anionic surfactants	mg kg^{-1}	117±16	91±9
total PAHs	µg kg^{-1}	2286±126	1672±103
total PCBs	µg kg^{-1}	11±0.6	11±0.6
total Phthalates	µg kg^{-1}	37528±2437	50047±2844

Online Resource 14. Concentration of conventional organic micropollutants in the feed sludges of UMT

		FU	FP
EOX	µgCl kg^{-1}	12000±857	13400±1100
total non-ionic surfactants	µg kg^{-1}	973±9	2264±21
total anionic surfactants	mg kg^{-1}	630±54	486±38
total PAHs	µg kg^{-1}	3631±158	2955±134
total PCBs	µg kg^{-1}	6.7±0.2	4.3±0.1
total Phthalates	µg kg^{-1}	49536±3895	53806±3478

Online Resource 15. Concentration of conventional organic micropollutants in the feed sludges of AA

		AA-FU (secondary)	AA-FU (mixed)
EOX	µgCl kg^{-1}	5040±280	4500±40
total non-ionic surfactants	µg kg^{-1}	1336±12	4402±35
total anionic surfactants	mg kg^{-1}	n.d.	1550±14
total PAHs	µg kg^{-1}	2423±105	3175±145
total PCBs	µg kg^{-1}	22±0.5	42±1.5
total Phthalates	µg kg^{-1}	86099±5000	57673±3550

Online Resource 16. Concentration of emerging micropollutants in the feed sludges of TT

		Test 1	
		TT-FU	TT-FP
QACs	µg kg^{-1}	30000±3800	35000±1300
Pharmaceuticals	µg kg^{-1}	120±8.1	160±5.3
Biocides and fungicides	µg kg^{-1}	2300±47	1700±160
		Test 2	
		TT-FU	TT-FP
QACs	µg kg^{-1}	26000±1000	30000±900
Pharmaceuticals	µg kg^{-1}	110±5.4	140±6.5
Biocides and fungicides	µg kg^{-1}	2800±430	1600±170

Online Resource 17. Concentration of emerging organic micropollutants in the feed sludges of UMT

		UMT-FU	UMT-FP
QACs	µg kg^{-1}	49000±1700	45000±3300
Pharmaceuticals	µg kg^{-1}	130±31	130±27
Biocides and fungicides	µg kg^{-1}	3300±1100	3600±390

Online Resource 18. Concentration of emerging organic micropollutants in the feed sludges of AA

		AA-FU(secondary)	AA-FU(mixed)
QACs	µg kg^{-1}	26000±2000	130000±6300
Pharmaceuticals	µg kg^{-1}	130±8.1	120±10
Biocides and fungicides	µg kg^{-1}	1400±110	3600±460

Online Resource 19: Results of biotests with sludge samples

Sample code and experiment	Inhibition of dehydrogenase activity in *A. globiformis,* EC_{50} [g kg^{-1}] [1]	Avoidance behaviour of earthworms (%) [2]
TT-FU_I	40 (35-48)	n.d.
TT-DU_I	30 (26-36)	n.d.
TT-FP_I	59 (49-71)	n.d.
TT-DP_I	217 (196-241)	n.d.
TT-FU_II	25 (14-36)	85 (9)
TT-DU_II	31 (19-43)	28 (23)
TT-FP_II	15 (4-25)	64 (15)
TT-DP_II	36 (25-46)	68 (23)
UMT-FU_I	*104 (69-194)*	36 (26)
UMT-MDU_I	405 (207-1231)	-37 (36)
UMT-MTDU_I	88 (67-127)	-6 (50)
UMT-FP_I	38 (23-93)	64 (22)
UMT-MDP_I	203 (139-335)	8 (47)
UMT-MTDP_I	110 (83-152)	-32 (39)
AA-FU_I	55 (44-70)	68 (27)
AA-DAA_I	373 (288-534)	52 (23)
AA-FU_II	*1 (0-3)*	n.d.
AA-DAA_II	19 (15-23)	n.d.
CAN_I	*1544 (800-4813)*	22 (30)
CAN_II	*889 (305-6729)*	-8 (42)

[1] *Arthrobacter globiformis* test (ISO 2012). Shown are estimated sample concentration causing 50% effect compared to quartz sand control (EC50), and their 95% confidence intervalls. Values in italic indicate extrapolated EC50. Measurements based on four biological replicates.
[2]Earthworm (*Eisenia fetida*) avoidance test (ISO 2008). Shown are mean avoidance responses (%) with their standard deviation tested at a concentration of 25 g sludge/kg soil d.w.

A.1.4 Assessing the ecological long-term impact of wastewater irrigation on soil and water based on bioassays and chemical analyses

Richter E, Hecht F, Schnellbacher N, Ternes TA, Wick A, Wode F, Coors A (2015): Water Research.

Accepted for publication July 8, 2015

Author contributions:

Initials: Elisabeth Richter (ER), Fabian Hecht (FH), Nadine Schnellbacher (NAS), Thomas A. Ternes (TT), Arne Wick (AW), Florian Wode (FW), Anja Coors (AC).

Study design and planning:

ER: 70%; AC: 20%; AW: 10%.

Performance of the experiments and analyses:

ER: 60%, construction and performance of soil column experiment, sampling, biololigal tests; NAS: 20%, biological tests; AW, FH, FW: 20%, chemical analyses.

Data assembling and preparing of figures:

ER: 70%, evalutation and preparation of graphs of biological and chemical results; AC: 20%, support in statistical evaluation; AW, FH, FW: 10%, assembling of chemical results.

Data analysis and interpretation:

ER: 60%, analysis and interpretation of biological and chemical results; ACO: 20%, AW, FH, FW: 20%, discussion and further ideas for analysing and interpreting biological and chemical data.

Introduction, results and discussion:

ER: 60%; AC: 20%; AW: 10%; TT: 10%.

ASSESSING THE ECOLOGICAL LONG-TERM IMPACT OF WASTEWATER IRRIGATION ON SOIL AND WATER BASED ON BIOASSAYS AND CHEMICAL ANALYSES

Elisabeth Richter [a, b], Fabian Hecht [c], Nadine Schnellbacher [a], Thomas A. Ternes [d], Arne Wick [d], Florian Wode [e], Anja Coors [a]

[a] ECT Oekotoxikologie GmbH, Flörsheim / Main, Germany

[b] Department of Aquatic Ecotoxicology, Goethe University Frankfurt am Main, Frankfurt / Main, Germany

[c] Department of Earth Sciences, Institute of Geological Sciences, Hydrogeology Group, Freie Universität Berlin, Berlin, Germany

[d] Federal Institute of Hydrology, Koblenz, Germany

[e] Berliner Wasserbetriebe, Labor, Berlin, Germany

* Corresponding author. Elisabeth Richter, ECT Oekotoxikologie GmbH, Böttgerstr. 2-14, 65439 Flörsheim / Main, Germany

Tel.: +49 6145 9564 61; E-mail address: e.richter@ect.de

Abstract:

The reuse of treated wastewater for irrigation and groundwater recharge can counteract water scarcity and reduce pollution of surface waters, but assessing its environmental risk should likewise consider effects associated to the soil. The present study therefore aimed at determining the impact of wastewater irrigation on the habitat quality of water after soil passage and of soil after percolation by applying bioassays and chemical analysis. Lab-scale columns of four different soils encompassing standard European soil and three field soils of varying characteristics and pre-contamination were continuously percolated with treated wastewater to simulate long-term irrigation. Wastewater and its percolates were tested for immobilization of *Daphnia magna* and growth inhibition of green algae (*Pseudokirchneriella subcapitata*) and water lentils (*Lemna minor*). The observed phytotoxicity of the treated wastewater was mostly reduced by soil passage, but in some percolates also increased for green algae. Chemical analysis covering an extensive set of wastewater-born organic pollutants demonstrated that many of them were considerably reduced by soil passage, particularly through peaty soils. Taken together, these results indicated that wastewater-born phytotoxic substances may be removed by soil passage, while existing soil pollutants (e.g. metals) may leach and impair percolate quality. Soils with and without wastewater irrigation were tested for growth of plants (*Avena sativa*, *Brassica napus*) and soil bacteria (*Arthrobacter globiformis*) and reproduction of collembolans (*Folsomia candida*) and oligochaetes (*Enchytraeus crypticus*, *Eisenia fetida*). The habitat quality of the standard and two field soils appeared to be deteriorated by wastewater percolation for at least one organism (enchytraeids, plants or bacteria), while for two pre-contaminated field soils it also was improved (for plants and/or enchytraeids). Wastewater percolation did not seem to raise soil concentrations of classical organic pollutants and priority substances, while a significant retention was found for zinc and several organic micropollutants, particularly in the peaty soils, thus matching these soils' observed higher removal efficiency. Overall, our results demonstrate that benefits of wastewater irrigation can come with the cost of deteriorating soil habitat quality and depend on the respective soil and considered test organism. The approach employed here represents a feasible tool to assess these integrated effects at lab-scale while being predictive for scenarios at field-scale.

Keywords:

Soil aquifer treatment; emerging pollutants; whole effluent toxicity testing; ecotoxicology; environmental risk assessment; soil quality.

Introduction

Urban areas of metropolises facing growing water scarcity together with increasing water demand could benefit from the reuse of treated wastewater on land (Lischeid *et al*., 2011). Moreover, this reuse could help to further improve the quality of treated wastewater and of receiving surface waters as demanded by the European Water Framework Directive (WFD, 2000). At a global scale, wastewater reuse by irrigation on land is widely practiced, particularly in arid areas (Hamilton *et al*., 2007). Concerns about wastewater irrigation relate to groundwater pollution and deterioration of the irrigated soil resulting from the accumulation of wastewater-born metals and organic pollutants (Munoz *et al*., 2009). However, soil contamination is often assessed with respect to risks for human health from crop consumption, while ignoring the potential impacts on soil habitat quality for terrestrial organisms (Prosser and Sibley, 2015). Environmental impacts of wastewater irrigation have been studied by monitoring individual wastewater-born pollutants in soil or water using laboratory or (semi-) field experiments (Ternes *et al.*, 2007, Munoz *et al*., 2009, Grossberger *et al*., 2014) or by measuring the removal efficiency of wastewater-born pollutants and pathogens via constructed wetlands, bank filtration or soil aquifer treatment (Verlicchi and Zambello, 2014). The focus of such studies has thus been on either water or soil quality but rarely addressed the trade-off between these two compartments (Munoz *et al*., 2009, Orias *et al.* 2013). Soil passage of treated wastewater can enhance the biodegradation of pollutants compared to the discharge into streams, however, adsorptive pollutants can also be retained in the soil and accumulate (Munoz et al., 2009). Selecting already contaminated instead of pristine land may relativize the pollution of soil by wastewater irrigation, but increase the risk for groundwater and surface water quality due to remobilization of old contaminants (Hamilton 2007, Lottermoser 2012). Usually, these risks are assessed by measuring already manifested effects during or *ex-post* studies at field-scale, but an experimental lab-scale method to estimate potential trade-offs *ex-ante* is missing.

Therefore, the present study aimed to assess in an integrative and predictive approach the impact of long-term irrigation with treated wastewater on the quality of soil as habitat for terrestrial organisms and the influence of soil passage on the water quality for aquatic organisms, thus considering both affected environmental compartments together. Saturated, packed soil columns were continuously percolated with treated wastewater in a condensed timescale to represent a worst-case scenario with regard to low degradation and high soil retention compared to field conditions. Moreover, the influence of soil characteristics in terms of physicochemical properties and existing precontamination was investigated. To this end, an aquatic and a terrestrial bioassay battery encompassing standard ecotoxicity tests were combined with comprehensive chemical monitoring of priority and emerging pollutants.

The ecotoxicological hazard of a complex environmental sample depends on the chemical composition and usually differs among exposed species due to their individual sensitivity toward the single components and their mixture. Therefore, a battery of aquatic and terrestrial bioassays was applied representing different trophic levels and toxicity endpoints, to assess the quality of treated wastewater, its percolates after soil passage and the respective soils. In the context of wastewater irrigation, percolate quality can be seen as indicative for the impact on groundwater, adjacent surface water as well as soil pore water. Applying aquatic biotest batteries to assess the hazard of treated wastewater (whole effluent toxicity testing, WET) is widely established (Power and Boumphrey, 2004, Orias *et al*., 2013) and follows standardised test guidelines. Green algae as unicellular primary producers and crustacean as primary consumers are regularly required in WET, whereas water lentils as macrophytic primary producers are less frequently used in this context although being particularly suitable for the testing of turbid eluates (Moser and Römbke, 2009). These aquatic biotests proved also appropriate to assess the ecotoxicity of waste materials' leachates (Krüger *et al.*, 2011), but have rarely been applied for the analysis of soil percolates (Abrantes *et al.*, 2008). Until now, terrestrial bioassays are not considered in European legislation on soil protection, despite their successful usage in characterizing the ecotoxicity of contaminated sites (Hawthorne *et al.*, 2005). The benefit and reliability of a terrestrial biotest battery such as that used in the present study was already demonstrated for the assessment of waste in a comprehensive international ringtest (Moser and Römbke, 2009). Assessing wastewater irrigated soil by applying biotests appears therefore promising, but has not been reported so far.

Materials and methods

Soil column apparatus:

An experimental apparatus was constructed taking into account the recommendations on soil column design of Lewis and Sjöstrom (2010) for saturated packed soil columns. The columns consisted of stainless steel tubes of 20 cm height and 22 cm diameter (6532 cm^3 volume for soil) and lids of polyoxymethylene. The soil was held between two fixed steel meshes (mesh size 1.0 mm) covered at each side by a sieve fabric disk (polyester, mesh size 0.25 mm, SEFAR AG, Switzerland). The columns were continuously percolated from bottom to top to ensure water saturation of the soil (further details are described in Text S.1).

Validation experiment with climbazole-spiked treated wastewater:

A four weeks percolation experiment was performed to demonstrate the suitability and reliability of the apparatus by percolating European standard soil (LUFA 2.3, LUFA, Speyer, Germany; identical with s4, Table 1,) at 9.0±0.1 kg soil d.w. per column (n=3 replicate columns) with treated wastewater

spiked with the fungicide climbazole. The percolation regime (6.9±0.1 mL/min, equivalent to 10.0 ±0.2 L/d, for 28 d) and spiking concentration (1 mg/L climbazole) were selected to yield a climbazole concentration of approximately 25 mg/kg soil d.w. (assuming 90% adsorption), expected to result in 50% effect in plant tests (Richter *et al.*, 2013). After percolation, the soil of each column was thoroughly homogenized. One soil sample per soil column was stored frozen (<5 months) and analyzed in triplicate for climbazole (method see Table S.1 in supplements). The remaining soil was air-dried and used in growth inhibition tests with *Avena sativa* and *Brassica napus* (ISO, 2012a). Each of the technical replicates (i.e. soil columns) was tested with four biological replicates (i.e. plant pots), in parallel with four replicates of untreated (i.e., non-percolated) LUFA 2.3 soil.

Main experiment with treated wastewater and four soils:

In order to experimentally simulate long-term irrigation (approximately 30 years assuming a realistic rate of 730 L/m^2*year, Ternes et al., 2007), four packed soil columns were continuously percolated with treated municipal wastewater (WW feed) for 77 days (6.9±0.1 mL/min, equivalent to 10.0±0.2 L/d or 276 L/m^2*d, approximately 54 cm/d filter velocity). Each of the four columns was packed with a different soil, encompassing one reference soil and three field soils representing various soil properties but also different levels of precontamination. The field soils were sampled at the study site Hobrechtsfelde, located close to Berlin, Germany, where raw wastewater had been irrigated onto almost 100 km^2 between the 1870s and the 1980s and where impacts on soil and groundwater quality have been extensively studied (Lottermoser *et al.*, 2012). For the last ten years, treated wastewater has been led into constructed wetlands and channels of this area in the course of pilot projects aiming to prevent falling water levels that are threatening forest and wetland biotopes and are promoting the remobilization of heavy metals from degraded organic matter (Lottermoser et al., 2012). Our experiment used treated wastewater from the same source and was performed *in situ* at the respective wastewater treatment plant (WWTP, with 575000 population equivalents, employing activated sludge treatment with iron phosphate precipitation followed by denitrification and secondary sedimentation). The following parameters were measured on average in the WW feed: temperature: 18.8°C (linear decline from 21°C to 15°C), pH 8.0, electrical conductivity: 1143 µS/cm, DOC: 11.3 mg/L, Ca^{2+}: 103 mg/L, Cl^-: 135 mg/L, NO_3^-: 42 mg/L, PO_4^{3-}: 0.5 mg/L and SO_4^{2-}: 145 mg/L. Redox conditions in the center of the soil columns were aerobic during the percolation period (350 mV to 600 mV). Break-through curves for conductivity determined in the first 24 h of the experiment confirmed homogenous percolation regimes for all columns.

Sampling and treatment of soil in the main experiment:

Field soils were collected from plots of approximately 2 m^2 from a depth of 10 cm-50 cm (i.e. omitting the vegetation layer). Soil s1 (N 52.66'161; E 13.48'133) was sampled at a long-term raw sewage farm bed that had later been limed to rise the pH and increase sorption and immobilization of metals. Soils s2 (N 52.65'527; E 13.47'400) and s3 (N 52.66'834; E 13.48'722) were sampled at locations with little influence of former raw wastewater irrigation, but potential input of current irrigation. Sampled soils were air-dried, homogenized, sieved (4 mm) and stored at 4°C. European standard soil, LUFA 2.3 was used as reference soil (s4). A batch of each soil was retained (i.e. not used in the percolation experiment) to serve as control in the bioassays. At the termination of percolation, the drained soil of each column was thoroughly homogenized. For each of the percolated and untreated soils, one representative sample (i.e. n=1) was taken and stored frozen (<8 months) until chemical analysis and determination of soil characteristics. The remaining batch of each homogenized soil was air-dried, sieved (4 mm) and stored at 4°C (<6 months) until use in terrestrial bioassays.

Soil characteristics such as grain size analysis, total organic carbon (TOC), maximum water holding capacity (WHK_{max}) and pH were determined by standard methods (see supplements S.2).

Sampling of percolates:

During three sampling periods (day 0-3, 30-33, and 72–75, referred to as sampling 1, 2 and 3) three 24 h composite samples were taken of the WW feed and each soil column percolate. The feed was always sampled 24 h earlier than the percolates to better achieve hydraulically corresponding samples. Sampling 1 was expected to be influenced primarily by substance-specific adsorption of wastewater-born substances and by a first flush of leaching of existing contaminants from the soils; it thereby represents the initial phase of a newly started irrigation. At samplings 2 and 3, adsorption, sequestration and degradation-related removal of wastewater-born substances was supposed to be at steady state, while leaching of existing contaminants would be mostly over; these samplings thus represent long-term irrigation. For the organic pollutants, the 24 h samples of each sampling period were analyzed individually (n=3 per sampling period and percolate/feed). For all other parameters and the biotests, the three 24 h composite samples were pooled for each sampling period (i.e., n=1 per sampling period and percolate/feed). Samples were passed through 0.45 µm cellulose-acetate filters and stored refrigerated (<3 weeks) or frozen (<5 months) in amber glass bottles until analysis (organic pollutants) or refrigerated (<1 months) in polyethylene bottles (anions) and acidified with 65% HNO_3 (cations). For the biotests, unfiltered samples were stored frozen (<14 months) until use.

Chemical analyses:

In the aqueous samples, more than 100 substances (substances listed on Annex X of the WFD, metals, emerging pollutants, pharmaceuticals, biocides, and transformation products) were analyzed. In the soil samples (1 g to 10 g, freeze-dried and ground, with residual pore water contributing to <10% of soil concentrations), more than 100 substances (PCBs, PAHs, quaternary ammonium compounds (QACs), tin organics, pharmaceuticals, pesticides, biocides, metals) were analyzed. Total soil metal concentrations were measured in nitric acid extracts after microwave digestion. Analyses of metals and classic organic pollutants (PCBs, PAHs and tin organics) were done in accordance to German (DIN) or European (EN) standard protocols without analytical replication. According to frequently analyzed reference materials standard deviations can be predicted to be <10% for metals and <20% for organic pollutants. Pharmaceuticals, biocides and QACs were measured in triplicate. Detailed protocols are available in supplements (Table S.1). All reported soil concentrations refer to soil dry weight (d.w.).

Calculations:

For the experiment with climbazole-spiked treated wastewater, a variance component analysis was performed (STATISTICA, version 10) using plant biomass and length per biological replicate as dependent variable and the technical replicates (3 columns) as random variable.

For the main experiment, the removal from WW feed by soil passage was calculated individually for substances detected in the feed above their limit of quantification (> LOQ). If values in the percolates were < LOQ, they were replaced by LOQ.

$$Removal\ [\%] = 100 - \frac{concentration\ in\ percolate}{concentration\ in\ WW\ feed} * 100 \qquad \text{Equation 1}$$

In order to estimate the theoretical toxicity, toxic units (TUs) were calculated for *Daphnia magna* and *Pseudokirchneriella subcapitata* by dividing the measured concentration in the sample by the respective EC_{50} and calculating the sum of all TU for each sample and test organism. A sum of TU exceeding 1 indicates that the sample is expected to exhibit an effect of at least 50%.

Biotest battery:

The biotests were selected following recommendations of the ISO guideline 15799 (ISO, 2003) and were conducted according to international test guidelines. The aquatic biotests included green algae (*P. subcapitata*, 72 h growth inhibition test, n=3 replicates, ISO 8692, 2012b) and water lentils

(*Lemna minor,* 7 day growth inhibition test, n=4 replicates, ISO 20079, 2005) as primary producers, and a freshwater crustacean (*D. magna,* 48 h immobilization test, n=4 replicates, ISO 6341, 2012c) as consumer. The percolates of one sampling campaign were always tested in parallel with all aquatic bioassays. Prior to test start, the pH of the percolates was adjusted to 8.0 ± 0.1 for *P. subcapitata* and *D. magna,* and to 5.7 ± 0.1 for *L. minor*. The samples were tested with 90% percolate concentration and 10% 10-fold concentrated growth medium (modified Steinberg medium) for *L. minor*, with 89% percolate concentration and 10% 10-fold concentrated growth medium (ISO medium) for *P. subcapitata,* and with undiluted percolate for *D. magna*. The results obtained for the percolates were expressed as percentage inhibition compared with the growth in the respective laboratory medium controls (*P. subcapitata, L. minor*) or as mortality (*D. magna*).

Significantly different groups within each sampling were identified using the Tukey HSD test for multiple comparison (α=0.05) in STATISTICA (version 10).

The terrestrial biotest battery employed six organisms to represent different trophic levels and exposure routes: oats (*Avena sativa*) as monocot and oilseed rape (*Brassica napus*) as dicot plant (16 d growth inhibition test, ISO 11269-2, 2012a), collembolans (*Folsomia candida,* 28 d reproduction test, ISO 11267, 2001) belonging to the arthropods, enchytraeids (*Enchytraeus crypticus,* 28 d reproduction test, ISO 16387, 2004) and earthworms (*Eisenia fetida*, 56 d reproduction test, ISO 11268-2, 2011) belonging to the oligochaetes, and an ubiquitous soil bacterium (*Arthrobacter globiformis,* 1 h enzyme activity inhibition test, draft ISO 18187, 2012d) as microorganism. All soil samples (i.e., soils before and after percolation) were always tested in parallel with each terrestrial bioassay. There were four (*A. sativa*, *B. napus*, *A. globiformis*, *E. fetida*) or five (*F. candida*, *E. crypticus*) replicates per treatment.

The Tukey HSD test (STATISTICA version 10) was used for multiple comparisons within each test system (α=0.05). Detailed biotest protocols are reported in Text S.3.

Results and discussion

Validation experiment with climbazole-spiked treated wastewater:

In the soil percolated with treated wastewater spiked with climbazole (1 mg/L), the inhibition of plant growth reached on average 61% for shoot length of both *B. napus* and *A. sativa* and 51% and 68%, respectively, for plant biomass, compared to growth in the untreated soil. These effects were in accordance with the expected phytotoxicity of a nominal climbazole soil concentration of approximately 25 mg/kg (Richter *et al.*, 2013), which would correspond to approximately 80% sorption during percolation. The analytically measured climbazole concentration in the percolated

soil was 8.2 ± 0.3 mg/kg soil d.w. and thus confirmed the retention of climbazole. The difference in biologically indicated and analytically measured soil concentration might be explained by the formation of non-extractable but still bioavailable residues in soil. The variance component analysis confirmed that technical replicates contributed only 7% (shoot length) and 22% (biomass) to the total variance, whereas the greater part of variance was explained by the biological replicates with 93% (shoot length) and 78% (biomass). Overall, these results demonstrate that the experimental design was in principle suitable to discover effects of toxic substances sorbed to soil after irrigation with wastewater. Moreover, it showed that technical replicates could be sacrificed for the benefit of testing different soils in parallel and that four biological replicates per soil were appropriate in the bioassays.

Changes of wastewater quality by soil passage assessed by biotests:

All conducted aquatic biotests with the WW feed and its percolates after soil passage were valid according to the criteria of the respective test guidelines (Table S.2).

In *L. minor*, the WW feed of sampling 1 caused no growth inhibition compared to the medium control, whereas moderate (30%) to strong growth inhibition (78%) was observed at sampling 2 and 3, respectively (Figure 1a). While at sampling 1, no difference was found between growth in the WW feed and the soil percolates, significantly improved growth compared to the WW feed was observed in the percolates of soils s2 and s3 at sampling 2 and in percolates of all four soils at sampling 3. In summary, soil passage generally reduced the toxicity of the WW feed toward *L. minor*, with the greatest reduction being observed for the peaty soils s2 and s3.

In *P. subcapitata*, the WW feed caused no growth inhibition at sampling 1, little effects at sampling 2 (18%) but high inhibition at sampling 3 (70%), similar to the findings for *L. minor* (Figure 1b). While passage through soil s2 did not alter the algal growth compared to the WW feed in any of the samplings, significantly improved growth compared to the WW feed was found at sampling 3 for the percolates of s1, s4 and particularly s3. However, at sampling 1 and 2, growth inhibition in the percolates of s3, s4 and particularly s1 was always significantly greater than in the WW feed. Hence, effects of the soil passage on the toxicity toward *P. subcapitata* depended on the sampling period and differed among the soils: While at the start and middle of the experiment, soil passage through s1, s3 and s4 led to a deterioration of the WW feed quality for algae, the same soils improved the WW feed quality at the last sampling.

Survival of *D. magna* in WW feed as well as in percolates of all samplings was at least 95% (data not shown). Thus, the WW feed quality for survival of crustaceans was generally adequate, and it was not impaired by soil passage, independently of their existing pre-contamination. It is known that the

sensitivity of crustaceans and primary producers can differ by orders of magnitude due to the specific mechanisms of toxic action, e.g. for pesticides (Abrantes et al., 2008), which may explain the difference observed here for *D. magna* and primary producers.

Phytotoxicity of the WW feed and its partial reduction by soil passage was indicated by both primary producers, *L. minor* and *P. subcapitata,* in a similar pattern. Nutrients were added in sufficient amounts to the samples before the test while no excessive leaching of nutrients by soil passage was observed, ensuring that nutrients were not responsible for the observed effects. Physicochemical parameters such as pH in WW feed and soil percolates were within the suitable range (Table S.3). Consequently, the presence of phytotoxic contaminants in the WW feed and their partial removal by soil passage appears to be the most likely explanation for the observed patterns. This is in agreement with Tang *et al*. (2014) who identified organic micropollutants present in treated municipal wastewater as the cause for growth inhibition of primary producers. Deterioration of water quality by passage through some of the soils was only indicated by *P. subcapitata* and will be discussed later on.

Removal of wastewater-born pollutants by soil passage:

Chemical analysis of the WW feed showed that the threshold values proposed for surface water bodies under the European Water Framework Directive, the average annual environmental quality standards (AA-EQS; EC, 2013), were met for all analyzed priority substances except for Zn (exceedance ≤ factor 6) and occasionally for Cu and Ni (exceedance ≤ factor 2) (Table S.4). Among the 92 analyzed organic pollutants, 45 were detected in the WW feed at all samplings, with the greatest concentrations (>2 µg/L) for benzotriazole, 4-formylaminoantipyrine (FAA), iopamidol, acesulfame, diatrizoate, diclofenac, and carbamazepine (Tables S.5-S.7).

Soil passage of the WW feed generally reduced the concentration of Zn, Cu and Ni to values more than factor 2 below the respective AA-EQS (Table S3), except for percolates of soil s1. The removal of organic wastewater-born pollutants ranged from zero to complete removal at the presumed steady-state conditions of the last two sampling periods (Table S.5). No considerable removal (≤20%) occurred for example for diatrizoate and iopamidol, and moderate to high removal e.g. for bezafibrate (66%) and atenolol (>90%), which is in line with findings from field studies (Ternes *et al.,* 2007, Verlicchi and Zambello, 2014).

In order to compare overall removal efficiency among sampling periods and among soils, the concentrations (in nmol/L) of the 45 detected organic pollutants were summed for each sample (Figure 2a). While this parameter is not indicative of environmental risk due to the lack of ecotoxicological data, it is assumed to be indicative for other, non-monitored substances with similar

behavior. In relation to the WW feed, this parameter was reduced by 74% to 86% in the percolates at test start and by 11% to 28% in the longer term. Grouping these 45 substances characterized by poor (<40%), moderate (40-70%) or high (i.e. >70%) removal indicated constant removal at the latter two samplings (Figure 2b, Table S.6), which supports the assumption that removal during this phase (week 4 to 11) is at steady-state and thereby representative on a long-term scale. During this late phase, the peaty soils (s2 and s3) exhibited significantly greater removal efficiency than the less peaty soils (s1 and s4): Moderate to high removal (≥41% removal) was observed for a higher proportion of the 45 organic pollutants in the peaty soils (43%) compared to the less peaty soils (33%; ANOVA, $p = 0.009$, $n=4$). The better removal may be related to the elevated TOC content and thus increased sorption capacity in these soils and will be discussed in 3.7 in the context with soil quality. Further, the acidic soil pH of the peaty soils may have increased removal because the fraction of protonated species of basic compounds (e.g. metoprolol) and that of neutral species of acidic compounds (e.g. diclofenac), which exhibit a higher sorption affinity than the corresponding neutral or negatively charged fractions, prevail at low pH (Schaffer et al., 2012).

Leaching of existing soil contaminants:

A risk of remobilizing existing pollutants was expected for the soils s1, s2 and s3, because these soils had been sampled at sites influenced by former raw wastewater irrigation as confirmed by contamination with metals, PAHs and PCBs (Table 2). Increased metal concentrations in the percolates compared to the WW feed (Table S.4) were detected after passage through soil s1 for Ni in all samplings (increase by factor 4 to 10) and for Cu only in sampling 1 (increase by factor 3), resulting in an exceedance of the respective AA-EQS threshold values by factor 2 to 8. Also in sampling 1, an increase was found for Al (soil s2) and for As (soil s4). While the assumed analytical error of metal determination is assumed low (<10%), the results have to be treated with care as they rely on one composite percolate sample per sampling period. However, the findings demonstrate that WW passage through contaminated soil can indeed result in metal leaching and impaired water quality particularly at the onset of irrigation. None of the existing pre-contamination of the soils with organic pollutants (PCBs, PAHs, biocides, tin organics, and QACs) appeared to be mobilized by wastewater percolation based on measured soil concentrations and considering the assumed analytical error of 20%. However, as these pollutants were not measured in the percolate samples, subtle but for water organisms relevant leaching cannot be excluded.

Linking aquatic biotests and chemical analyses:

A theoretical approach to rank the detected substances according to their toxic potential and predict their combined effects is the calculation of the sum of toxic units (sum TUs) based on the measured concentration and toxicity of individual pollutants (Orias 2013, Verlicchi and Zambello, 2014, Leusch *et al*., 2014, Tang *et al*., 2014). In the present study, for only 19 of the detected organic substances toxicity estimates (as EC_{50}, the median effective concentration) for *Daphnia* and green algae could be retrieved from literature to calculate TUs (Tables S.8 and S.9).

For the survival of *D. magna,* the sums of TU were below 0.002 in all samples, indicating that no effects were to be expected from the combination of these 19 pollutants; this was not contradicted by our observations. For *P. subcapitata*, the sums of TUs ranged from 0.018 to 0.174, being well below the value of 1 that would predict 50% inhibition. Among these substances, the antibiotics clarithromycin and erythromycin and the herbicides (also used as biocides) diuron, atrazine and terbutryn contributed most to the algal sum TUs. The environmental relevance of these herbicides has been pointed out before as their removal in WWT is poor (Köck-Schulmeyer *et al.* 2013) and they often dominate negative effects on algae in WW effluents (Tang *et al.* 2014). The antibiotics clarithromycin, erythromycin and sulfamethoxazole have previously been reported to potentially pose a risk to aquatic organisms in treated wastewater, also after its subsequent passage through constructed wetlands (Verlicchi and Zambello, 2014). For *L. minor*, EC_{50} values were only available for diuron, atrazine, mecoprop, climbazole, erythromycin and sulfamethoxazole (Table S.7) and their sum of TUs reached at most 0.272 in a given sample, which does not explain the observed effects. The TU approach based on the few pollutants for which it was feasible could not sufficiently explain the observed phytotoxicity. Yet, the simple sum concentrations of detected organic substances (Figure 2a) showed a significant positive correlation with the growth inhibition of *L. minor* (Pearson $r^2 = 0.75$, $p = 0.01$, $n = 15$), but not with that of *P. subcapitata*. While this is no proof of causality, it implies that effects in *Lemna* were indeed linked to pollutants present in the WW feed, which were removed by the soil passage. Presumably, the remaining organic pollutants for which no toxicity estimates were available and other substances that had not been included in the analytical monitoring may have contributed to the overall phytotoxicity. This conclusion agrees with the statement that predicted effluent toxicity based on analytical measurements is often lower than the measured response in biotests (Leusch *et al*., 2014).

Metals present in the samples may have added to phytotoxic effects. Particularly concentrations of Zn in the WW feed were in the range of reported EC_{50} for *P. subcapitata* and *L. minor* (van Sprang *et al.*, 2009, Naumann *et al.*, 2006, Table S.4). However, a quantitative estimation of the contribution of metals to the overall phytotoxicity is hampered by considerable uncertainty as metal toxicity strongly depends on speciation and complexation so that reported EC_{50} for the same species and endpoint may

vary widely depending on the test conditions. While this can be taken into account in biotic-ligand models, it is currently not possible for all metals and test organisms. Overall, it demonstrates that for municipal wastewater comprising an unknown number of substances, it is hardly possible with current knowledge to attribute observed effects in a bioassay to individual pollutants among a limited set of measured substances. Including e.g. *in vitro* bioassays specific for distinct modes of action such as genotoxicity, carcinogenicity, dioxin-like activity, or estrogenic activity (Power and Boumphrey, 2004) could support the detection of further, more subtle acting substances.

In summary, an overall positive effect of the soil passage on WW feed quality was indicated by both the observed reduction in phytotoxicity to water lentils and the average removal of wastewater-born organic substances from the WW feed, with the peaty soils s2 and s3 tending to be most efficient. On the other hand, a deterioration of the WW feed quality by soil passage was indicated by the increase in toxicity toward algae as well as by the increase in metal concentrations at the first two samplings, pointing at an initial leaching of pollutants from contaminated soils (particularly s1).

Impact of wastewater percolation on soil quality assessed by biotests:

All conducted terrestrial biotests with the untreated and the WW percolated soils were valid according to the validity criteria of the respective guidelines regarding the untreated soil s4 as reference (Table S.2, Figure 3). The untreated soils as such possessed adequate habitat quality for all test organisms with the exception of soil s1 (the most pre-contaminated soil) that exhibited high mortality and little reproduction of *E. crypticus*.

Compared to the respective untreated soil, the percolation with WW significantly deteriorated the habitat quality of s2 for *A. globiformis,* of s3 for *A. sativa*, and of s4 for *E. crypticus* (Figure 3). In contrast, WW percolation significantly improved the habitat quality of s1 for *E. crypticus,* and of s2 for both plant species. No significant effects of the WW percolation were observed for *F. candida* and *E. fetida* in any of the soils. This may indicate that these two organisms were more tolerant than the other test organisms to the pollutants already present or accumulated after percolation in the here tested soils; however, their importance in representing soil dwelling decomposers as well as their sensitivity e.g. for waste materials (Moser and Römbke, 2009) renders these bioassays still relevant. Overall, species-specific effects are the reason why a battery of biotests covering a wide range of trophic levels, taxonomic groups and toxicity endpoints is recommended for the assessment of complex samples such as wastewater and contaminated soil (Hamilton et al., 2007, Römbke and Moser, 2009). The increase in pH of the initially acidic soils s2 and s3 as only reason for the observed negative effects can be excluded because the soil pH was in all case within the tolerance ranges of the test organisms, and the organisms performed well in soils with similar pH in these tests.

Other soil parameters such as the TOC were either not altered or did not change to unfavorable conditions regarding the test organisms' requirements. This renders it probable that retention of wastewater-born pollutants on the one hand and mobilization and leaching of existing pollutants on the other hand were responsible for the observed changes in soil habitat quality.

Impact of wastewater percolation on soil quality as indicated by chemical analyses:

The untreated soils s2 and s3 were similar with regard to their rather high TOC and low pH (Table 1), whereas the untreated soils s1 and s4 were both less acidic and poor in TOC. WW percolation resulted in a pronounced increase of soil pH in s2 and s3 (up to neutral pH), but no change in s1 and s4. The TOC was not altered by WW percolation by more than 10%, while soil texture was not expected to change by percolation and therefore not assessed. Several contaminants exceeded German regulatory precautionary threshold values (BBSchV, 1999) in the untreated soils, particularly in s1 and s2 (Table 2), which confirms the characterization of these soils as pre-contaminated. In the European standard soil LUFA 2.3 (s4), threshold values were approached by Cu and slightly exceeded by Zn.

Measured soil concentrations of metals and already present organic pollutants did not indicate any leaching due to WW percolation (Table 2). However, an initial leaching which does not affect the soil concentration within the assumed analytical error in soil of 20% can be relevant for percolate quality as demonstrated by the increased Ni and Cu concentrations in the percolates that were in the range of respective AA-EQS values. Hence, soil measurements need to be accompanied by percolate measurements for a sensitive detection of relevant leaching processes.

A substantial (>20%) increase in the concentration of already present contaminants due to WW percolation was observed for Zn in all soils (increase by up to 220%) and for Cu and Ni in s3 (by 50% and 45%, respectively). For Zn, the increase was in accordance with its partial removal from the aqueous phase (Table S.4). Hence, our percolation experiment indicated that prolonged irrigation with treated wastewater can lead to an accumulation at least of Zn in soil, which is in line with field study results (Hamilton et al., 2007). Also for benzo(a)pyrene, an increase (by 62%) was observed in s4. However, as measured soil concentrations were close to benzo(a)pyrene's LOQ and no increase was found for the related sum parameter PAHs, the significance of the increase appears weak.

Accumulation due to WW percolation was indicated for 21 emerging organic pollutants (Table S.10A), which were not detected in the untreated soils. Of these 21, eight occurred at concentrations >5 µg/kg soil d.w. in at least two soils after percolation (Figure 4), generally increasing with increasing average concentrations in the WW feed. Soil concentrations of these eight substances were always greatest in the most carbon-rich soil s3 and a positive but weak correlation was detected

with soil TOC (Spearman rank correlation, R = 0.416, p = 0.008, n = 40), which agrees with findings from wastewater irrigated field soils (Kinney *et al.*, 2006). However, soil TOC could not fully explain the differences in concentrations among the four soils, because after normalisation to TOC soil fraction (Table S.10B), differences were smaller but still significant (2-way ANOVA for factors soil and substance, both $p < 0.005$). This indicates that additional soil parameters such as pH or cation exchange capacity influenced retention, or that soil-specific degradation occurred.

Soil accumulation and persistence potential is known for carbamazepine from soils irrigated with treated wastewater (Dalkmann *et al.*, 2014, Grossberger *et al.*, 2014) and can be assumed for benzotriazole due to high effluent concentrations and low biodegradability (Reemtsma *et al.*, 2010). Also for trimethoprim and diclofenac, detection in wastewater irrigated soils has been reported from field studies (Dalkmann et al., 2014, Grossberger et al., 2014), while metoprolol has been detected in crops irrigated with treated wastewater (Prosser and Sibley, 2015). The agreement of our findings (Figure 4) with this available literature demonstrates that the design and setup of our experiment was suitable to simulate and detect removal and accumulation of wastewater-born pollutants in irrigated soil on a long-term scale. Our study presumably underestimated removal of wastewater-born pollutants by soil passage, because realistic field conditions are expected to enhance sorption due to longer hydraulic retention times while it may have overestimated sorption due to increased microbial degradation at longer time scale under field conditions. Yet, accumulation in the biologically relevant top layer of the soil (first 5 to 10 cm), the habitat of most soil organisms (Henzler *et al*., 2014), was underestimated due to the mixing of the soil of the whole column.

Linking terrestrial biotests and chemical soil analyses:

Two of the three observed negative impacts of WW percolation on soil habitat quality (for *A. globiformis* and *A. sativa*) were indicated for the same soils that also showed the greatest retention of wastewater-born organic pollutants (s2 and s3). This supports the conclusion that pollutant residues still present in treated wastewater can sorb to soil during wastewater irrigation and can lead to negative effects for soil organisms. Due to the scarce data on terrestrial ecotoxicity of organic pollutants, however, calculation of toxic units to identify potentially responsible substances was not feasible. Of some importance for the observed negative effects could have been the metals Cd and Zn, as their measured concentrations particularly in soil s1 were in the range of reported effective concentrations for *E. crypticus* (EC_{50} of 6 mg Cd/kg and 40 mg Zn/kg, Gomes *et al.*, 2014) and for plants (toxicity of Zn observed at >100 mg/kg and ≤300 mg/kg, Montilla *et al.*, 2003). However, measured soil concentrations of these metals did not change in a way that was consistent with the reduction of effects in soil s1 observed after percolation.

Instead, the improving effect of the WW percolation on the habitat quality of soils s1 and s2 for enchytraeids and plants, respectively, suggested that the percolation reduced the level of existing toxic contaminants by mobilization and leaching. A reduction in the existing pre-contamination of the soils was indirectly demonstrated for the metals Cu and Ni by percolate analysis, but similarly, pre-existing organic pollutants may have undergone subtle losses. Even changes that affect not the total but only the bioavailable soil concentration of pollutants can alter their biological effects, as described by Hawthorne *et al.* (2005) for the reduction of enchytraeids mortality in PAH-contaminated soils after mild extraction of soil. Also other authors confirmed that the terrestrial ecotoxicity of complex samples rarely correlate with total measured soil concentrations of contaminants, because the toxicity is determined by the bioavailable contaminant fraction which depends on soil properties (Römbke and Moser, 2009). Hence, terrestrial biotests are an indispensable tool for assessing the habitat quality of soil.

Conclusions

The employed experimental set-up of percolating soil columns with treated wastewater yielded removal rates as well as soil concentrations of selected wastewater-born pollutants mostly similar to those reported in field studies. This supports the conclusion that results from the lab-scale approach can be seen predictive for prolonged irrigation with wastewater at field scale. In addition, the employed aquatic and terrestrial biotest battery appears as a suitable tool to assess long-term effects of wastewater irrigation on soil and water quality. Chemical analysis demonstrated that carbon-rich and acidic soils (s2 and s3) were more efficient than sandy and basic soils in removing wastewater-born pollutants but at the same time retained greater concentrations of numerous emerging organic pollutants and zinc. Consistently, some biotests pointed at a removal of toxic, particularly phytotoxic, substances from the wastewater as well as a deterioration of soil quality after percolation particularly in peaty soils. For soils with existing pre-contamination, increased algal toxicity and metal concentrations in water after soil passage and decreased effects in soils after percolation provide indirect evidence for remobilization and leaching of existing pollutants from these soils, particularly at the start of the irrigation. Results from bioassays and chemical analyses generally led to similar conclusions regarding the impact of soil passage on the quality of treated wastewater and of percolated soils. Yet, the ecotoxicity found in the samples could not be predicted based on aqueous or soil concentrations of the measured pollutants. The effects observed in the biotests were not similar among soils, which will be due to soil-specific retention and leaching of the individual pollutants. They were also not uniform among test organisms, which will be due to species-specific sensitivity to the individual pollutants. This supports previous recommendations of assessing complex environmental samples such as wastewater and contaminated soil by a broad range of

biotests. A valuable complement to the here employed *in vivo* test battery would be a set of *in vitro* bioassays to better detect pollutants with a highly relevant and specific mode of action such as genotoxicity, but their application was beyond the scope of the present study. Based on the phytotoxicity observed in the investigated wastewater and in percolated soils, we recommend that chemical monitoring in wastewater irrigation studies should include a range of herbicides and metals encompassing their bioavailability as they may be highly relevant to explain ecotoxic effects. Overall, the present study clearly demonstrated that improving the quality of treated wastewater by soil passage comes at the price of deteriorated soil quality in terms of habitat suitability for terrestrial organisms and retention or even accumulation of pollutants. Water management and land use concepts involving irrigation with treated wastewater should take into account that selecting uncontaminated soils with high sorption potential, e.g. rich in organic matter, will be beneficial for groundwater protection, but consequently lead to a more pronounced impairment of these soils with previously high quality. Using instead pre-contaminated land will increase the risk of remobilization of existing pollutants by wastewater irrigation and thereby the deterioration of water quality. Therefore, an environmental impact assessment of wastewater irrigation should be performed on a case-by-case basis, considering the properties of the individual wastewater and the soil to be irrigated.

Acknowledgements

The authors thank J. Oehlmann, E. Topp, U. Kunkel, U. Dünnbier, R. Gnirss, G. Lischeid and all ELaN project partners for valuable discussions. This study was financially supported by the German Federal Ministry of Education and Research through the project ELaN (FKZ: 033L025F).

References

Abrantes, N., Pereira, R., Soares, A.M.V.M., Gonçalves, F., 2008. Evaluation of the ecotoxicological impact of the pesticide Lasso® on non-target freshwater species, through leaching from nearby agricultural fields, using terrestrial model ecosystems. Water Air and Soil Pollution 192, 211–220.

BBodSchV, 1999. Bundes-Bodenschutz- und Altlastenverordnung vom 12. July 1999. BGBL I, Nr. 36, 1554-1582.

Dalkmann, P., Siebe, C., Amelung, W., Schloter, M., Siemens, J., 2014. Does long-term irrigation with untreated wastewater accelerate the dissipation of pharmaceuticals in soil? Environmental Science and Technology 48, 4963–4970.

EC 2013: Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy.

Gomes, S.I.L., Scott-Fordsmand, J.J., Amorim, M.J.B., 2014. Profiling transcriptomic response of Enchytraeus albidus to Cu and Ni: Comparison with Cd and Zn. Environmental Pollution 186, 75–82.

Grossberger, A., Hadar, Y., Borch, T., Chefetz, B., 2014. Biodegradability of pharmaceutical compounds in agricultural soils irrigated with treated wastewater. Environmental Pollution 185, 168–177.

Hamilton, A.J., Stagnitti, F., Xiong, X., Kreidl, S.L., Benke, K.K., Maher, P., 2007. Wastewater irrigation: The state of play. Vadose Zone Journal 6, 823–840.

Hawthorne, S. B., Lanno, R. and Kreitinger, J. P. (2005). Reduction in acute toxicity of soils to terrestrial oligochaetes following the removal of bioavailable polycyclic aromatic hydrocarbons with mild supercritical carbon dioxide extraction. Environmental Toxicology and Chemistry 24, 1893–1895.

Henzler, A.F., Greskowiak, J., Massmann, G., 2014. Modeling the fate of organic micropollutants during river bank filtration (Berlin, Germany). Journal of Contaminant Hydrology 156, 78–92.

ISO (International Organisation for Standardization), 2001. Soil Quality - Effects of soil pollutants on Collembola (*Folsomia candida*). Methods for the determination of effects on reproduction. ISO 11267. Geneve, Switzerland.

ISO (International Organisation for Standardization), 2003. Soil Quality – Guidance on the ecotoxicological characterization of soils and soil materials. ISO 15799. Geneve, Switzerland.

ISO (International Organisation for Standardization), 2004. Soil Quality - Effects of pollutants on Enchytraeidae (*Enchytraeus sp*.). Determination of effects on reproduction. ISO 16387. Geneve, Switzerland.

ISO (International Organisation for Standardization), 2005. Water quality – Determination of toxic effect of water constituents and waste water to duckweed (*Lemna minor*) – Duckweed growth inhibition test. Geneve, Switzerland.

ISO (International Organization for Standardization), 2012a. Soil quality – determination of the effects of pollutants on soil flora. Part II: Effects of chemicals on the emergence and growth of higher plants. ISO 11269-2. Geneve, Switzerland.

ISO (International Organization for Standardization), 2012b. Water quality – freshwater algal growth inhibition test with *Desmodesmus subspicatus* and *Pseudokirchneriella subcapitata*. ISO 8692. Geneve, Switzerland.

ISO (International Organization for Standardization), 2012c. Water quality – determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) – Acute toxicity test. ISO 6341. Geneve, Switzerland.

ISO (International Organisation for Standardization), 2012d. Soil quality – Quality of solid samples - Solid contact test using the dehydrogenase activity of *Arthrobacter globiformis*. Draft guideline ISO 18187. Geneve, Switzerland.

ISO (International Organization for Standardization), 2011. Soil quality – effects of pollutants on earthworms (*Eisenia fetida*). 11268-2. Geneve, Switzerland.

Kinney, C.A., Furlong, E.T., Werner, S.L., Cahill, J.D., 2006. Presence and distribution of wastewater-derived pharmaceuticals in soil irrigated with reclaimed water. Environmental Toxicology and Chemistry 25, 317–326.

Leusch, F.D.L., Khan, S.J., Gagnon, M.M., Quayle, P., Trinh, T., Coleman, H., Rawson, C., Chapman, H.F., Blair, P., Nice, H., Reitsema, T., 2014. Assessment of wastewater and recycled water quality: A comparison of lines of evidence from in vitro, in vivo and chemical analyses. Water Research 50, 420–431.

Lewis, J., Sjöstrom, J., 2010. Optimizing the experimental design of soil columns in saturated and unsaturated transport experiments. Journal of Contaminant Hydrology 115, 1–13.

Köck-Schulmeyera, M., Villagrasab, M., López de Aldaa, M., Céspedes-Sánchezc, R., Venturac, F., Barcelóa, D., 2013. Occurrence and behavior of pesticides in wastewater treatment plants and their environmental impact. Science of the Total Environment (458–460), 466–476.

Krüger, O., Kalbe, U., Richter, E., Egeler, P., Römbke, J., Berger, W., 2013. New approach to the ecotoxicological risk assessment of artificial outdoor sporting grounds. Environmental Pollution 175, 69–74.

Lischeid, G., Nathkin, M., 2011. The Potential of Land-Use Change to Mitigate Water Scarcity in Northeast Germany – a Review. DIE ERDE – Journal of the Geographical Society of Berlin 142, 97–113.

Lottermoser, B.G., 2012. Effect of long-term irrigation with sewage effluent on the metal content of soils, Berlin, Germany. Environmental Geochemistry and Health 34, 67–76.

Moser H., Römbke J., 2009. The ecotoxicological characterisation of waste – results and experiences from a European ring test. New York, Springer Ltd., 308.

Montilla, I., Parra, M. A., Torrent, J., 2003. Zinc phytotoxicity to oilseed rape grown on zinc-loaded substrates consisting of Fe oxide-coated and calcite sand. Plant and Soil 257, 227–236.

Muñoz, I., José Gómez -Ramos, M., Aguera, A., Francisco García -Reyes, J., Molina-Díaz, A., Fernández-Alba, A.R., 2009. Chemical evaluation of contaminants in wastewater effluents and the environmental risk of reusing effluents in agriculture. Trends in Analytical Chemistry 28, 676–694.

Naumann, B. Eberius, M., Appenroth, K.J., 2007. Growth rate based dose–response relationships and EC-values of ten heavy metals using the duckweed growth inhibition test (ISO 20079) with Lemna minor L. clone St. Journal of Plant Physiology 164 (12), 1656–1664.

Orias, F., Perrodin, Y., 2013. Characterisation of the ecotoxicity of hospital effluents: A review. Science of the Total Environment 454–455, 250–276.

Power, E.A., Boumphrey, R.S., 2004. International trends in bioassay use for effluent management. Ecotoxicology 13, 377–398.

Prosser, R.S., Sibley, P.K., 2015. Human health risk assessment of pharmaceuticals and personal care products in plant tissue due to biosolids and manure amendments, and wastewater irrigation. Environ. Int. 75, 223–233.

Reemtsma, T., Miehe, U., Duennbier, U., Jekel, M., 2010. Polar pollutants in municipal wastewater and the water cycle: Occurrence and removal of benzotriazoles. Water Research 44, 596–604.

Schaffer M., Boxberger N., Börnick H., Licha T., Worch E., 2012. Sorption influenced transport of ionizable pharmaceuticals onto a natural sandy aquifer sediment at different pH. Chemosphere 87(5), 513-20.

Tang, J.Y.M., Busetti, F., Charrois, J.W.A., Escher, B.I., 2014. Which chemicals drive biological effects in wastewater and recycled water? Water Research 60, 289–299.

Ternes, T.A., Bonerz, M., Herrmann, N., Teiser, B., Andersen, H.R., 2007. Irrigation of treated wastewater in Braunschweig, Germany: An option to remove pharmaceuticals and musk fragrances. Chemosphere 66, 894–904.

Verlicchi, P., Zambello, E., 2014. How efficient are constructed wetlands in removing pharmaceuticals from untreated and treated urban wastewaters? A review. Science of the Total Environment 470–471, 1281–1306.

Tables and Figures:

Table 1: Key parameters of the four soils (s1, s2, s3, s4) after percolation with treated wastewater for 77 days (WW) and untreated (CON).

	s1	**s2**	**s3**	**s4**
Clay [%]	5	11	12	24
Silt [%]	3	5	5	15
Sand [%]	86	79	76	60
Soil texture (USDA)	loamy fine sand	sandy loam	sandy loam	sandy clay loam
WHCmax [g/100 g]	31.1	61.3	66.1	37.3
Soil mass per column [kg d.w.]	9.5	5.8	4.7	8.5
TOC [%], CON	1.8	5.5	10.0	1.1
TOC [%], WW	1.7	5.8	9.3	1.1
pH, CON	6.8	4.8	5.5	6.9
pH, WW	7.0	6.4	6.7	7.0

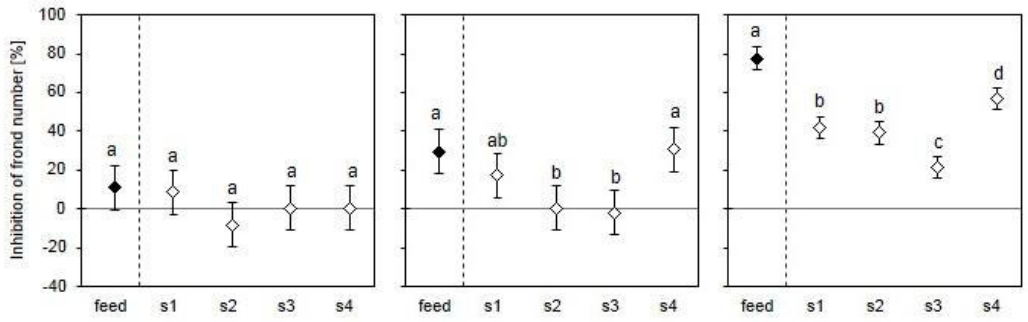
Clay = <0.02 mm; silt = 0.02 mm – 0.063 mm; sand = >0.063 mm; TOC: total organic carbon; WHC_{max}: maximum water holding capacity; pH measured in 0.01 M $CaCl_2$ (n=3).

Table 2: Concentrations of major contaminants in the four soils (s1, s2, s3, s4) after percolation with treated wastewater (WW) and untreated (CON) together with regulatory thresholds (VW) and limit of quantification (LOQ), in mg/kg soil dry weight.

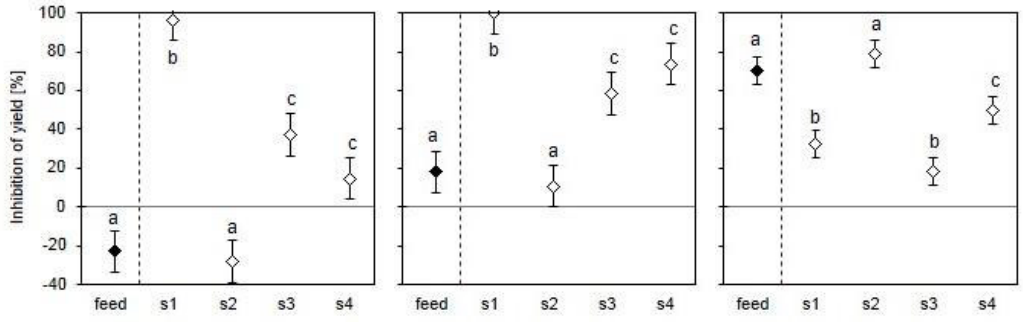
	CON				**WW**				**VW**[a]	LOQ
	s1	**s2**	**s3**	**s4**	**s1**	**s2**	**s3**	**s4**		
Al	6700	5500	4300	9300	6300	5300	4200	10000		10
As	<5	17	<5	7	<5	18	<5	7	2	5
Cd	5.0	0.43	0.10	0.19	5.1	0.40	0.14	0.18	0.4	0.1
Cr	139	19	6	21	151	20	7	23	30	1
Cu	55	26	5	19	61	29	8	21	20	1
Ni	14	4	3	11	15	5	5	12	15	1
Pb	59	39	18	30	75	44	20	29	40	1
Zn	163	81	21	70	201	116	68	90	60	1
Hg	0.72	0.35	<0.01	<0.01	0.81	0.52	<0.01	<0.01	0.1	0.01
[b] PCB_6	0.27	<0.01	<0.01	<0.01	0.29	<0.01	<0.01	<0.01	0.05	0.01
Benzo(a)pyren	0.12	0.08	<0.01	0.04	0.14	0.08	<0.01	0.06	0.30	0.01
[c] PAH_{16} (EPA)	5.0	3.2	0.29	2.1	5.7	3.2	0.30	2.2	3.0	0.01
Triclocarban	0.24	<0.01	<0.01	<0.01	0.23	<0.01	<0.01	<0.01		0.01
DDT	0.06	0.35	<0.01	0.06	0.06	0.26	<0.01	0.04		0.01

[a] VW: German regulatory precautionary threshold values to ensure the maintenance of soil functions (referring to sand with <8% organic matter, BBodSchV, 1999), or regional background value (for As in Berlin area, from Lottermoser *et al.* 2012). [b] PCB_6 = Trichlorbiphenyl, Tetrachlorbiphenyl, Pentachlorbiphenyl, Hexachlorbiphenyl, Hexachlorbiphenyl, Heptachlorbiphenyl; [c] PAH_{16} = Naphthalene, Acenaphthylene, Acenaphthene, Fluorene, Phenanthrene, Anthracene, Fluoranthen, Pyrene, Benz(a)anthracene, Chrysene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(a)pyrene, Indeno(1,2,3-cd)pyrene, Dibenzo(a,h)anthracene, Benzo(g,h,i)perylene. Percolation with treated wastewater for 77 d at a rate of 276 $L/m^{2*}d$. Single measurements (n=1).

a: *L. minor*



b: *P. subcapitata*



Sampling 1 **Sampling 2** **Sampling 3**

Figure 1: Results of the aquatic biotests with WW feed (black diamonds) and 3 days-composite samples of percolate (white diamonds) from four different soils (s1, s2, s3, s4) obtained at start, middle and end of the column experiment (sampling 1, 2, 3). (a) Inhibition of frond number in *L. minor* in relation to medium control; (b) Inhibition of yield biomass in *P. subcapitata* in relation to medium control. Depicted are means and 95% confidence intervals (n = 4 (a) and 3 (b)). Homogenous groups within each sampling campaign are indicated by identical letters (Tukey HSD test, $p < 0.05$).

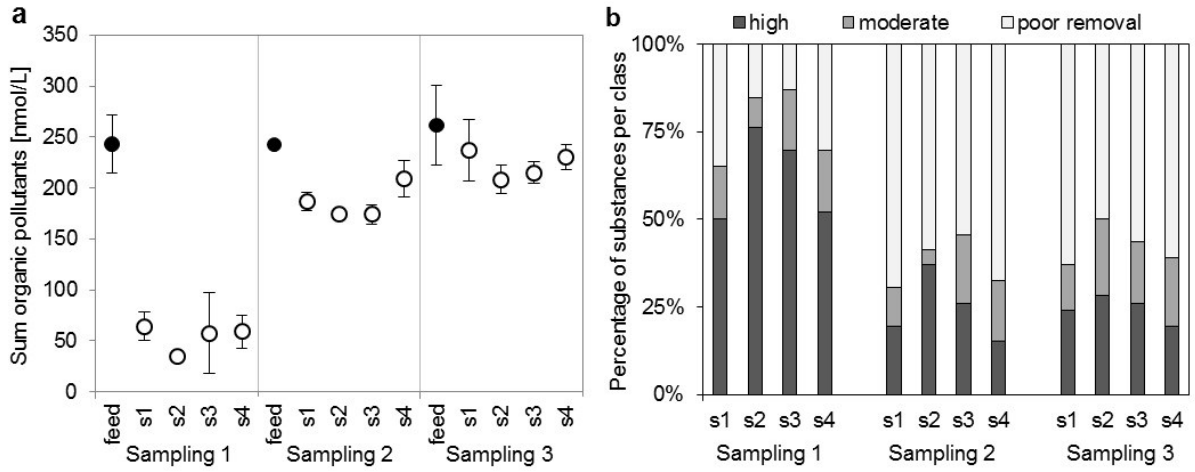


Figure 2: General occurrence of organic pollutants in WW feed and percolates after passage through columns with four different soils (s1, s2, s3, s4) at three samplings (start, middle, end of experiment). (a) Mean sum of molar concentrations (n = 3) of 45 substances with standard deviations. (b) Percentage of 45 substances in percolates falling into the classes of poor (≤40%), moderate (41%-70%) and high (71%-100%) removal by soil passage.

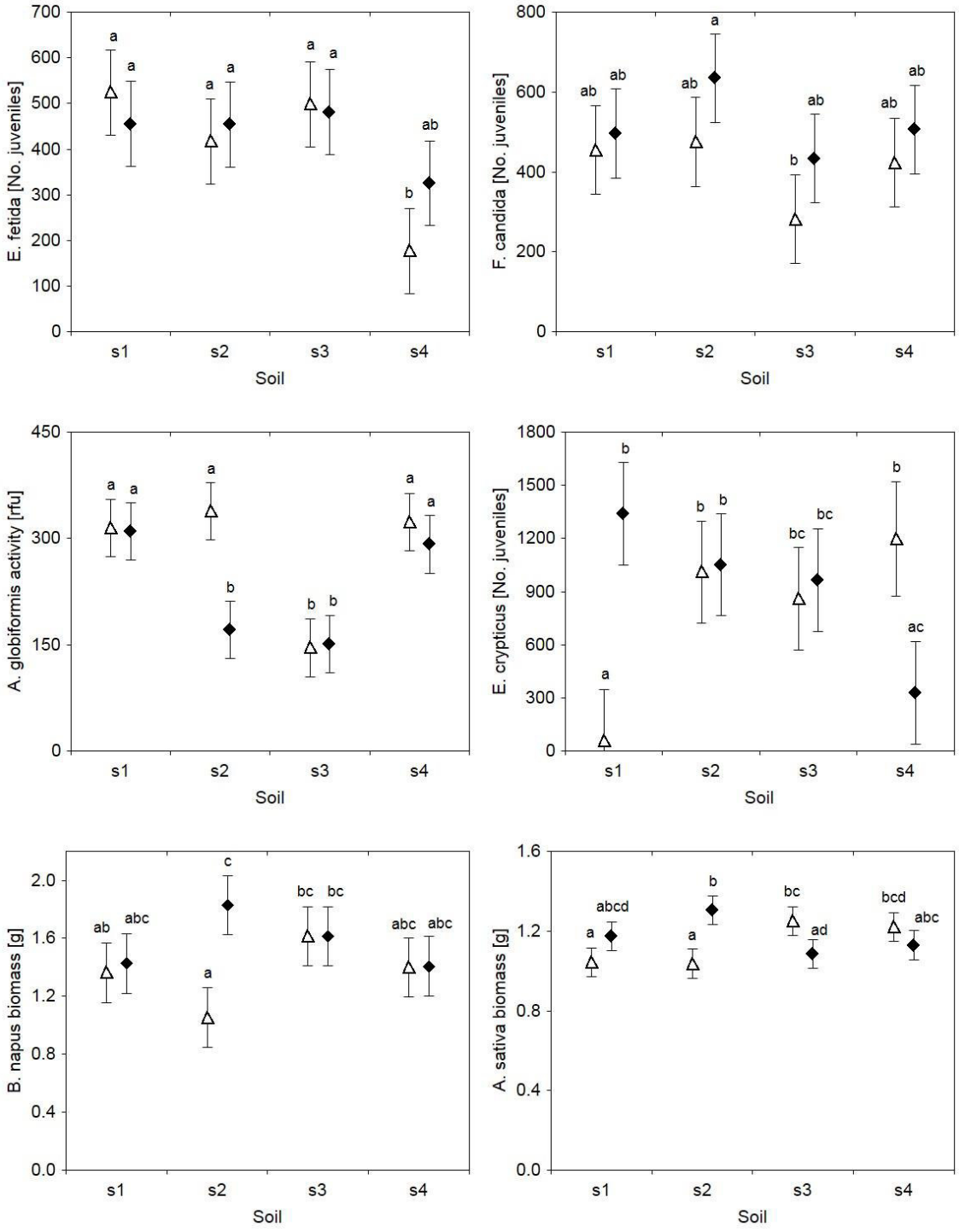


Figure 3: Results of the terrestrial biotests with four different soils (s1, s2, s3, s4) either untreated, i.e. stored cool for 77 days (CON, triangles) or percolated for 77 days with treated wastewater (WW, black diamonds). Shown are means and 95% confidence intervals (n = 4 for *A. sativa*, *B. napus*, *A. globiformis*, *E. fetida* and n = 5 for *F. candida* and *E. crypticus*). Soil s4 CON (untreated LUFA 2.3) served additionally as laboratory control. Homogenous groups are indicated by identical letters (Tukey HSD test, α = 0.05).

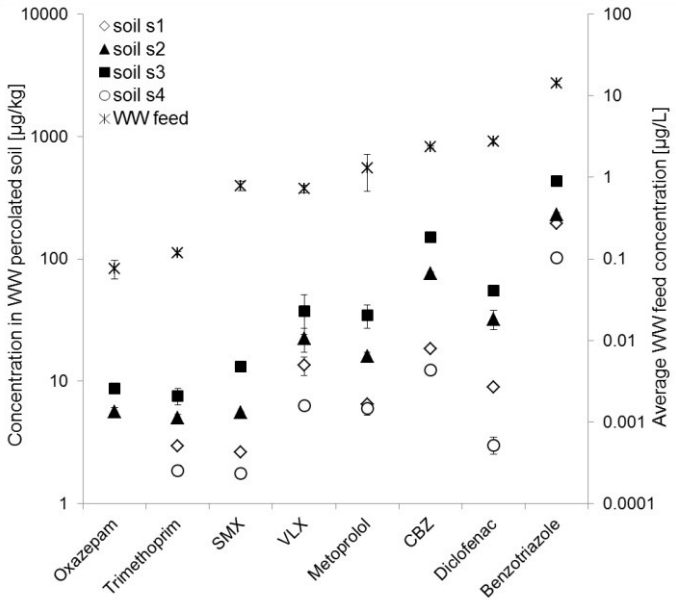


Figure 4: Soil concentrations of organic pollutants in four different soils (s1, s2, s3, s4) after percolation with treated wastewater (WW feed) in comparison to the average concentration in the WW feed. The substances were not present in the respective untreated soils and were detected in at least two of the WW percolated soils at > 5 µg/kg (LOQ in soil ≤ 0.5 µg/kg). Shown are means with 95% confidence intervals (n = 3 for soils and 9 for WW feed). SMX = sulfamethoxazole; VLX = venlafaxine; CBX = carbamazepine.

Supplementas to '*Assessing the ecological long-term impact of wastewater irrigation on soil and water based on bioassays and chemical analyses'*.

Text S.1: Details on column construction:

The columns of one treatment were connected to a storage container (stainless steel, volume of 30 L) via feed tubes of fluorinated ethylene propylene (FEP), teflon and tygon. The storage container was continuously fed by a stainless steel storage tank or directly by the treated wastewater pipe (connected via PVC tubes). The feeding regime (see below) was maintained by a peristaltic pump (IPC24, Ismatec, Wertheim-Mondfeld, Germany). The wastewater-bearing tubes were replaced after four weeks and the storage container and water filter were cleaned regularly. To ensure water saturation of the soil, the columns were percolated from bottom to top, and the percolates were collected in stainless steel vessels (10 L) separately for each column. The apparatus was protected from light to avoid biofilm growth in the tubes. The packing of the columns was done in intervalls to receive homogeneous distribution. After compaction and before adding a new layer of a few centimeters, the column was saturated from the bottom to ensure hydraulic connectivity. The packing procedure was spread over several days with time left for the soil to settle. In order to assess the leaching pattern of the columns, the break-through based on the conductivity in the percolates of all eight columns was monitored during the first 24 h of the experiment. During the experiments the redox potential at the center of each soil column (Redox electrode GE 105, Greisinger electonic GmbH) and the temperature of the feed and percolates were recorded continuously.

Text S.2: Soil characterisation:

The soil pH was measured in 0.01 M $CaCl_2$ according to DIN ISO 10390:2005 using a 1:5 volumetric relation of dry soil to water (n=3). The maximum water holding capacity (WHK_{max}) was determined according to DIN ISO 11268-2 annex D (2011). TOC in the freeze-dried soil samples was analyzed in accordance to DIN EN 13137 with an ELTRA Helios C/S Anlayzer (ELTRA GmbH, Haan, Germany) after dry combustion with subsequent infrared detection (n=3). Inorganic carbon of the sample was released prior to the IR-detection through acidifying the sample with hydrochloric acid. Grain-size analyses were carried out by a cascade of sieves with mesh sizes of 2000 µm, 630 µm, 200 µm, 63 µm and 20 µm. Dry-sieving was used to gain the <2000 µm and <630 µm fractions while wet-sieving in an ultrasonic bath was used for collecting the 200 µm, 63 µm and 20 µm fractions according to the procedure described in Wetzel et al. (2013).

(Wetzel, M.A., Winterscheid, A., Wahrendorf, D.-S., 2013. Baseline of the butyltin distribution in surface sediments (0-20 cm) of the Elbe estuary (Germany, 2011). Marine Pollution Bulletin 77, 418-423).

DIN EN 13137:2001: Characterization of waste - Determination of total organic carbon (TOC) in waste, sludges and sediments; German version, German Standardization Organization.

DIN ISO 10390, 2005: Bodenbeschaffenheit - Bestimmung des pH-Wertes, DIN Deutsches Institut für Normung e.V., Berlin, Germany.

Table S.1: Detailed methods and materials for chemical analyses:

S.1 A: Analyses in WW feed and percolates after soil passage (one measurement per sample):

Substance:	No of samples analysed per sampling period / LOQ:	Protocol:
Phenazone, 4-acetoaminoantipyrine (AAA), 1-acetyl-1-methyl-2-dimethylox-amoyl-2-phenyl-hydrazide (AMDOPH), 1-acetyl-1-methyl-2-phenylhydrazide (AMPH), 4-formylaminoantipyrine (FAA), 2-ethyl-2-phenyl-malonamide (PEMA), fenuron	1 (3 days composite) / ≤100 ng/L	The organic micropollutants were analyzed by an ultra high performance liquid chromatography - high resolution mass spectrometry multiresiduemethod, which is described in detail in Wode et al., 2012 (Wode, F.; Reilich, C.; van Baar, P.; Duennbier, U.; Jekel, M.; Reemtsma, T., 2012. Multiresidue analytical method for the simultaneous determination of 72 micropollutants in aqueous samples with ultra high performance liquid chromatography - high resolution mass spectrometry. J Chromatogr A, 1270, 118-126). Ultra high performance liquid chromatography (UPLC) on a reversed phase column with methanol-water gradient elution was used for chromatographic separation. An injection volume of 1 mL was enriched by online-solid phase extraction on a C18 Hypersil Gold column (12 µm, 20 mm x 2.1 mm ID, Thermo Fisher Scientific, Bremen, Germany) and separated on an ACQUITY UPLC HSS T3 column (1.8 µm, 50 mm x 2.1 mm ID, Waters, Manchester, UK). One complete run lasted 15 min, the same LC method was used for screening and quantification. The used mass spectrometer was a single stage Orbitrap Exactive PlusTM (Thermo Fisher Scientific, Bremen, Germany) with a maximum resolution of 140000 and mass deviation typically below 2 ppm. Switching between positive (ESI+) and negative electrospray ionization mode (ESI-) in Full Scan was employed. The resolution was 35000, the mass range 103-900 m/z for both ESI- and ESI+ mode.
Amphetamine, atrazine, desphenylchloridazone	1 (3 days composite) / ≤50 ng/L	The organic micropollutants were analyzed by a LC-MS/MS method, using a Xevo TQS (Waters, Manchester, UK). The LC analysis was performed on a Waters Acquity ultra-performance liquid chromatography system (Waters, Manchester, UK). Separation was performed on an Acquity HSS T3 column (100 mm × 2.1 mm, 1.7 µm particle size, Waters, Manchester, UK). The mobile phase was constituted by solvent A (0.1% formic acid in water) and solvent B (methanol). The flow rate was 0.4 mL/min with a linear gradient at the following conditions: 0–0.5 min, 95% A; 0.5–4.1 min, 5% A; 4.1–5.0 min, 5% A; 5.1–6.0 min, 95% A. Total run time was 6 min and the injection volume was 50 µL in full loop mode. The column temperature was set to 40 °C, the samples were cooled at 5°C. The LC system was coupled to a Waters Xevo TQ-S mass spectrometer (Manchester, UK) with an electrospray ionization interface. Waters MassLynx version 4.1 was used for LC/MS system control and data analysis. The desolvation gas was set to 1000 L/h at a temperature of 600°C, the source temperature was set to 150°C. The collision gas flow was set to 0.15 mL/min. Polarity switching between positive and negative ionization mode was used. The capillary voltages were set to 3.5 kV (ESI+) and -0.7 kV (ESI-). Data aquisition was done in multiple reaction monitoring (MRM) mode. All parameters were automatically optimized by MassLynx/IntelliStart. Auto dwell time was used to ensure that approximately 12 data points were acquired for each chromatographic peak. Quantification of the analytes was done using Target Lynx software (Waters, Manchester, UK).

Table S.1 A (continued):

Substance:	No of samples analysed per sampling period / LOQ:	Protocol:
Cations (Ca, Na, K, Mg, Fe, Mn, Ba, Sr, Li, B, Al, Zn, Pb, Cu, Ni, Cd, Co, Cr)	1 (3 days composite) / ≤5 µg/L	The collected samples stored in 50 ml polyethylene bottles were preserved refrigerated at 4°C until day of analysis. Cations were analysed with ICP-OES method (inductively coupled plasma optical emission spectrometry) using Optima 2100 PerkinElmer (PerkinElmer Inc., Waltham, MA, USA). The level of quantification (LOQ) for all cations was 0.002 mg/L, except for the ions K and Na with a LOQ of 0.2 mg/L and Al with 0.1 mg/L. The cations Hg, Sb and As were analysed using a flow injection analysis system coupled with atomic absorption spectroscopy (FIAS AAS) method on a PerkinElmer (PerkinElmer Inc., Waltham, MA, USA) with a LOQ of 0.005 mg/L and an injection volume of 500 µl.
Anions (Cl^-, NO^{3-}, $SO4^{2-}$, $PO4^{3-}$, Br^-)	1 (3 days composite) / 100 µg/L	Anions were analysed with ion chromatography (IC) method using Dionex ICS 1100 (Dionex GmbH, Idstein, Germany) with a LOQ of 0.1 mg/L and an injection volume is 25 µl. DOC and silicium (Si) content were analysed photometrically with Technicon Autoanalyser (Technicon AutoAnalyserII, Braun+Lübbe GmbH, Norderstedt, Germany) with a LOQ of 0.5 mg/L and an injection volume of 4.5 mL.
Other organic pollutants, including climbazole from validation experiment	3 (24 hours composite) / 5 ng/L - 200 ng/L	Samples were filtered through 0.45 µm filters made of regenerated cellulose and stored frozen (-25°C) until the day of analysis. Analyses were done by LC-MS/MS in accordance to a method described in Rühmland et al., 2015. Briefly, after addition of the labelled surrogate mix (200 ng/L final concentration), 80 µL of the samples were injected into an Agilent 1260 Series liquid chromatography system (Agilent, Waldbronn, Germany) coupled to an AB SCIEX TripleQuad™ 5500 mass spectrometer (Applied Biosystems, Darmstadt, GermanyFor chromatographic separation an Agilent Zorbax Eclipse XDB-C18 column (2.1 x 150 mm, 3.5 µm) equipped with a guard cartridge was used. Mobile phase A consisted of 0.1% formic acid. Methanol with 0.1% formic acid served as mobile phase B. A gradient elution from 100% mobile phase A to 98% mobile phase B was applied. The flow rate was set to 0.3 mL/min and the column temperature to 30°C. The MS system was operated with electrospray ionization (ESI) in the positive and negative ionization mode. For polarity switching, the settling time was set to 50 ms. The Scheduled MRM™ algorithm (detection window 80 s, target scan time 0.2 s, pause time 3 ms) was used for monitoring at least two mass transitions for each analyte. An internal standard calibration in ultrapure water was prepared for quantification. Matrix effects were compensated by the use of labeled surrogate standards. The limit of quantification (LOQ) was derived from the signal to noise (S/N) ratio in the samples. At the LOQ, the S/N ratio of one transition (quantifier ion) had to be at least 10 and that of the second transition (qualifier ion) at least 3. The calculated concentrations were only assessed valid if the ratio of values obtained for the quantifier and qualifier ion were in the range of 0.75 to 1.20. The accuracy and precision of the method was checked within each measurement series by recovery experiments (n ≥ 3) and repeated injections of reference samples.

S.1. B: Analyses in WW percolated and untreated soils (one sample per homogenised soil batch):

Substance:	No of measurements per sample/LOQ:	Protocol:
Emerging organic micropollutants, including climbazole from validation experiment	3 / ≤5 µg/kg	Freeze-dried and ground soil samples (< 2 mm) of 2 g were extracted in triplicate with pressurized liquid extraction (PLE) using an ASE-200 (Dionex, Idstein, Germany). Previous to extraction a labelled surrogate mix was added to the soil sample (5 ng/g). The extraction was done using four PLE cycles (80°C, 100 bar, flush of 120%) with a mixture of methanol and water (50:50; v/v). The extract was filled up with water to a final volume of 50 mL. An aliquot of 1 mL was then analyzed by LC-MS/MS as described above for the percolate samples.
QACs	3 / ≤15 µg/kg	Freeze-dried and ground soil samples (< 2 mm) of 2 g were extracted in triplicate with pressurized liquid extraction (PLE) using an ASE-350 (Dionex, Idstein, Germany). Previous to extraction a labelled surrogate mix was added to the soil sample (3.75 ng/g). The extraction was done using four PLE cycles (80°C, 100 bar, flush of 120%,) with 100% methanol. The extract was filled up with methanol to a final volume of 30 mL. An aliquot of 1 mL was then analyzed by LC-MS/MS as described in detail in Braguglia et al., 2015).
Polychlorinated biphenyls (PCBs) and other semi-volatile halogenated compounds (SVOC) and PAHs	1 / 10 µg/kg	SVOC and PAHs were analyzed according to the slightly modified standard protocols prEN 16167:2010 and prEN 16181:2010, respectively. Briefly, freeze-dried, ground and sieved (< 2000 µm) soil samples of 10 g were extracted by pressurized liquid extraction (PLE) using an ASE-200, (Dionex, Idstein, Germany). The extraction was done twice, each with 40 ml of a mixture of iso-hexane, acetone and n-heptane (62:33:5; v/v/v). Subsequently a mixture with surrogate standards was added containing 20 13C-labelled PCBs, HCHs, DDXs and 8 deuterated PAHs. Sample extracts were concentrated to 5 ml using a Büchi Synchore evaporator (BÜCHI, Konstanz, Germany) at 40 °C. The extracts were subjected to a sandwich column clean-up with desactivated aluminum oxide (Al2O3) and freshly activated copper powder for sulfur removal. Afterwards a GPC – cleanup was accomplished and the final sample volume was reduced to 0.5 ml before GC-MS/MS analysis (Chromtech Evolution Idstein Germany).
Tin	1 / 1 mg/kg	Tin organics were analyzed in 5 g of non-dried and homogenized soil sample according to DIN 19744 using an Agilent 6890N gas chromatograph (Agilent, Waldbronn, Germany) hyphenated to an OI Analytical 5380 pulsed flame-photometric detector (OI Analytical, College Station, Texas).
Other metals	1 / ≤1 mg/kg	The nitric acid soluble fraction of metals were extracted from freeze-dried and ground soil samples of 1 g by microwave digestion according to DIN EN 16173. All metals except for mercury were analyzed in the extracts using an ICP-OES (Perkin Elmer Optima 8300, Shelton, USA). Mercury was analyzed according to DIN 1483 using a mercury analyzer (Analytik Jena Mercur, Jena, Germany).

Remarks:

C M Braguglia, A Coors, A Gallipoli, A Gianico, E Guillon, U Kunkel, G Mascolo, E Richter, T A Ternes, M C Tomei, G Mininni. Quality assessment of digested sludges produced by advanced stabilization processes. Environ Sci Pollut Res (2015) 22:7216–7235.

S Rühmland, A Wick, TA Ternes, M Barjenbruch. Fate of pharmaceuticals in a subsurface flow constructed wetland and two ponds Ecological Engineering (2015) 80: 125–139.

Note: Excluded for the calculation of removal from WW feed and retention in soil were the compounds climbazole, propiconazole and terbutryne, because cross-contamination seemed to occurr from the used material of the column apparatus. However, they concentrations in the aqueous and soil samples were below effective concentrations for the test organisms so that their influence is assumed to be negligible.

Text S.3: Detailed methods and materials for bioassays

Inhibition of growth of water lentils after seven days (ISO 20079, 2005):

According to ISO 20079, *L. minor* was maintained as batch culture in modified Steinberg medium (ISO 20079) at 24 ± 2°C under continuous photosynthetically active radiation (PAR) of 85 – 135 µE/m2 × s. The same conditions applied to the bioassay. The samples were tested with 90% percolate concentration and 10% 10fold of concentrated growth medium. Samples and control were run with 4 replicates in crystallising dishes containing 150 mL test volume and 3-4 *Lemna* colonies (9 fronds for test with percolates of sampling 3, and 12 fronds for tests with percolates from samplings 1 and 2). After 7 days, frond number and frond dry weight were recorded and biomass yield and growth rate of frond number were evaluated as endpoints.

Growth inhibition of green algae after 72 h (ISO 8692, 2012):

P. subcapitata were maintained as batch cultures in ISO medium (ISO 8692) at 21 - 24°C under continuous PAR of 60 – 120 µE/m2 × s. The same conditions applied to the bioassay. The samples were tested with 89% percolate concentration and 10% of 10fold concentrated growth medium and 1% algal inoculum. Samples and control were run with 3 replicates and 1 blank in Erlenmeyer flasks containing 20 mL test volume. Test solutions were inocculated with cell densities of 1×10^4 cells/mL. After 72 h, cell density as surrogate parameter for biomass was estimated via autofluorescence measurements of 200 µl aliquots at the spectral fluorometer after substracting the respective blank fluorescence (Tecan multiplate reader, Tecan Group).

Mortality of water fleas after 48 h (ISO 6341, 2012):

D. magna were kept in Elendtmedium M4 (Elendt, B.-P., Bias, W.-R., 1990. Trace nutrient deficiency in Daphnia magna cultured in standard medium for toxicity testing. Effects of the optimization of culture conditions on life history parameters of D. magna. Water Research 24, 1157–1167) and subjected to a 16:8 light:dark cycle at low light conditions (~ 450 lx); temperature was maintained at 22 ± 1°C. The same conditions applied to the bioassay. The samples were tested with 100% and 50% percolate concentration. Samples and control were run with 4 replicates (each encompassing 5 daphnids) in glass beakers containing 20 ml of test solution. No feed was supplied and oxygen saturation was 100% throughout the test. Immobility after 48 h was evaluated as surrogate parameter for mortality.

Parameters of the aquatic bioassays are summarised in the following:

Sample / percolate from soil column	sampling	pH at test start (adjusted)	pH at test end	Conductivity [µS/cm]	Hardness [mmol/L]	Alkalinity [mmol/L]	Ammonium [mg/L]
Parameters in tests with water lentils (*L. minor*)							
control	1	5.7	7.3	898	--	0.3	0.01
WW feed	1	5.7	7.6	1307	--	1	0.42
s1	1	5.7	7.3	1150	--	0.8	3.28
s2	1	5.7	7.4	947	--	0.8	0.35
s3	1	5.7	--	--	--	--	--
s4	1	5.7	7.6	1286	--	0.8	0.20
control	2	5.7	7.2	922	--	1.5	0.01
WW feed	2	5.7	7.6	1136	--	1.2	0.13
s1	2	5.7	7.6	1152	--	1.3	0.47
s2	2	5.7	7.5	1202	--	1.1	0.56
s3	2	5.7	7.6	1205	--	1	0.22
s4	2	5.7	7.5	1232	--	0.9	0.31
control	3	5.7	6.7	914	--	0.5	0.00
WW feed	3	5.7	7	1339	--	0.6	0.05
s1	3	5.7	7.4	1377	--	1	0.12
s2	3	5.7	7.4	1311	--	0.7	0.18
s3	3	5.7	7.3	1391	--	0.8	0.18
s4	3	5.7	7.2	1230	--	1.2	0.11
Control in L. minor was modified Steinberg medium (ISO 20079).							
Parameters in tests with green alga (*P. subcapitata*)							
control	1	8.0	8.0	201	1.3	0.9	4.66
WW feed	1	8.0	8.6	792	2.4	2.7	0.30
s1	1	8.0	8.1	721	3.0	2.5	2.50
s2	1	8.0	8.0	642	2.2	0.6	0.29
s3	1	8.0	8.0	--	--	1.4	--
s4	1	8.0	8.3	937	2.7	3	0.18
control	2	8.0	7.0	1811	0.7	0.9	4.85
WW feed	2	8.0	8.7	712	1.8	1.6	0.03
s1	2	8.0	8.4	924	2.9	2.9	0.39
s2	2	8.0	8.5	536	1.4	1.9	0.32
s3	2	8.0	8.2	570	1.7	1.7	0.04
s4	2	8.0	8.3	766	2.3	2.3	0.21
control	3	8.0	7.1	1811	2.6	1.4	3.94
WW feed	3	8.0	8.5	1037	2.5	4	0.06
s1	3	8.0	8.6	872	3.4	3.5	0.11
s2	3	8.0	8.6	1351	3.5	4.6	0.21
s3	3	8.0	8.6	903	2.4	3.6	0.25
s4	3	8.0	8.6	1156	3.0	4.4	0.12
Control in P. subcapitata was ISO medium (ISO 8692).							

Parameters of the aquatic bioassays (continued):

Sample / percolate from soil column	sampling	pH at test start (adjusted)	pH at test end	Conductivity [µS/cm]	Hardness [mmol/L]	Alkalinity [mmol/L]	Ammonium [mg/L]
Parameters in tests with water fleas (*D. magna*)							
control	1	8.0	7.8	694	2.9	1.1	0.01
WW feed	1	8.0	8.1	792	2.4	2.7	0.30
s1	1	8.0	8.2	721	3.0	2.5	2.50
s2	1	8.0	8.1	642	2.2	0.6	0.29
s3	1	8.0	7.9	--	--	1.4	--
s4	1	8.0	8.3	937	2.7	3.0	0.18
control	2	8.0	7.9	673	3.0	1.9	0.00
WW feed	2	8.0	8.1	712	1.8	1.6	0.03
s1	2	8.0	8.3	924	2.9	2.9	0.39
s2	2	8.0	8.0	536	1.4	1.9	0.32
s3	2	8.0	8.0	570	1.7	1.7	0.04
s4	2	8.0	8.2	766	2.3	2.3	0.21
control	3	8.0	7.8	716	3.5	1.3	0.02
WW feed	3	8.0	8.4	1037	2.5	4.0	0.06
s1	3	8.0	8.3	872	3.4	3.5	0.11
s2	3	8.0	8.5	1351	3.5	4.6	0.21
s3	3	8.0	8.3	903	2.4	3.6	0.25
s4	3	8.0	8.4	1156	3.0	4.4	0.12

Control in D. magna was medium Elendtmedium M4.

Hardness was determined using Aquamerck 8039 test kit.

Growth inhibition of terrestrial plants after 14 days (ISO 11269-2, 2012):

According to ISO 11269-2, the soils were tested with certified seeds of turnip (*Brassica napus, Liforum, LUFA*) and oats (*Avena sativa,* St. Champion, SAATEN-Union GmbH) with 4 replicate pots. Before test start, the soil moisture was adjusted (to WHC_{max} of approximately 47% (s1), 55% (s2), 108% (s3), and 35% (s4)) and the substrates were distributed to the pots at approximately 107 to 346 g dry weight. Per pot, 10 seeds were sown and after determining the germination rate, seedlings were reduced to 5 representive plants. Plants were cultivated in a controlled environment chamber with a 16:8 light:dark cycle under approximately 230 $\mu E/m^2*s^1$ PAR; temperature was maintained at 24 ± 2°C; watering was provided as necessary and a liquid fertiliser was supplemented once. After 14 days, the endpoints germination rate (emergence), shoot length and shoot freshweight (biomass) were evaluated.

Inhibition of reproduction of enchytraeids after four weeks (ISO 16387, 2004):

According to ISO 16387, *Enchytraeus crypticus* was cultured in-house on agar at 20 ± 5°C and weekly fed oatmeal ad libitum. Synchronised adults were used for the test. Before test start, the soil moisture was adjusted (to WHC_{max} of approximately 50% (s1), 57% (s2), 54% (s3), and 48%(s4)) and readjusted weekly. The soils were tested with 5 replicate glass vessels containing 20 ± 5 g dry weight and 10 adult worms. Oatmeal was supplemented as food at 50 mg per replicate at test start and thereafter with 25 mg per week. The test was conducted in a controlled environment chamber with a 16:8 light:dark cycle under 400 to 800 lux at 20 ± 4°C. After 28 days, adult and juvenile worms were counted under the binocular after Bengal red staining over night; as endpoint, reproduction (number of juveniles) was evaluated.

Inhibition of reproduction of springtails after four weeks (ISO 11267, 2001):

According to ISO 11267, the collembolan *Folsomia candida* was cultured in-house on plaster of Paris (calcium sulphate with activated charcoal) in the dark at 20 ± 4°C; synchronised juveniles of 9 to 10 days age were used for the test. Before test start, the soil moisture was adjusted (WHC_{max} of approximately 38% (s1), 46% (s2), 34% (s3), 48% (s4)) and readjusted weekly. The soils were tested with 5 replicate glass vessels, containing 30 ± 5 g fresh weight and 10 collembolans. Yeast was supplemented as food with approximately 5 mg per glas vessel at test start and day 14. The test was conducted in a controlled environment chamber in the dark at 20 ± 4°C. After 28 days, adult and juvenile collembolans were counted under a binocular after ink staining; as endpoint, reproduction was evaluated.

Inhibition of reproduction of earthworms after eight weeks (ISO 11268-2, 2011):

According to ISO 11268-2, the earthworm *Eisenia fetida* was cultured in-house on a peat- cow manure substrate. For the test, adult worms with clitellum, an age of 2 to 12 months and with a fresh weight of 300 to 600 mg were used. Before test start, 10 g ground cow manure per kg soil dry weight was mixed into the soils as food and soil moisture was adjusted (WHC_{max} of approximately 55% (s1), 61% (s2), 105% (s3), and 43% (s4)). The soils were tested with 4 replicate vessels (with each 10 adult worms), containing 200 to 516 g dry weight depending on the soil in Bellaplast boxes covered by a transparent lid. Food was supplemented weekly for the first four weeks (5 g cow manure moistened with 10 mL water); soil moisture was readjusted weekly throughout the test. The test was conducted in a controlled environment chamber with a 16:8 light:dark cycle under 400 to 800 lux at 20 ± 4°C. After 28 days, the adult worms were removed, and survival and freshweight were recorded. After 56 days, the number of juvenile worms was determined by heat extraction in a waterbath.

Inhibition of dehydrogenase activity in soil bacteria (draft ISO 18187, 2012):

For the test according to draft ISO 10871 with the soil bacterium *Arthrobacter globiformis,* the soils were sieved (2 mm mesh size) and adjusted to 20% relative soil moisture. In addition, a positive control at 600 mg/kg Benzyldimethyl-hexadedyl-ammonium chloride (BAC, CAS 122-18-9, Sigma-Aldrich) on quarz sand was tested, which should result in 40 - 60% inhibitory effect. The soils were weighed into translucent microtiter plates (24-well plate, Greiner Bio-One GmbH, Frickenhausen, Germany) with 0.6 g freshweight per well and four replicates per soil. Plates were pasteurised twice for 15 minutes, bacteria inoculum was added at 0.4 mL per well and plates were incubated at 30 ± 2°C for 2 h on a horizontal shaker in the dark. Resazurin solution (CAS 62758-13-8, 4.5 mg/L, Sigma-Aldrich) was added with 0.8 mL per well, and fluorescence of the generated resofurin was measured for 60 minutes in 15 minute intervals using a spectral fluorometer (excitation at 535 nm, adsorbance at 590 nm). As endpoint, dehydrogenase activity was determined based on the fluorescence slopes between 15 and 45 minutes.

Table S.2: Compliance of the controls of the bioassays with the validity criteria of the respective test guidelines.

Test organism	Test guideline	Validity criteria for the controls:	Observed in test
Column experiment I (relating to untreated soil LUFA Sp2.3)			
A. sativa	ISO 11269-2, 2012	≥ 70% emergence in each pot	95%
		≥ 90% mean survival of emerged plants	100%
B. napus	ISO 11269-2, 2012	≥ 70% emergence in each pot	90%
		≥ 90% mean survival of emerged plants	100%

Table S.2 (continued)

Test organism	Test guideline	Validity criteria for the controls:	Observed in test
Column experiment II (referring to aquatic test media control or s4 CON, i.e. untreated LUFA Sp2.3)			
L. minor	ISO 20079, 2005	frond number doubling time < 2.5 days	1.8
P. subcapitata	ISO 8692, 2012	increase of cell number between 0 and 72 h > factor 16	286
		mean section-by-section growth rate < 35%	21%
		coefficient of variation of mean specific growth rate from 0 to 72 h < 7%	2%
D. magna	ISO 6341, 2012	immobilised daphnids after 48 h < 10%	0%
		dissolved oxygen concentration after 48 h ≥ 3 mg/L	≥ 9.6 mg/L
A. sativa	ISO 11269-2, 2012	≥ 70% emergence in each pot	95%
		≥ 90% mean survival of emerged plants	100%
B. napus,	ISO 11269-2, 2012	≥ 70% emergence in each pot	92%
		≥ 90% mean survival of emerged plants	100%
A. globiformis	draft ISO 18187, 2012	mean fluorescence increase from 0 to 60 min > factor 5	10
		inhibition by reference substance BAC (CAS 122-18-9) between 30 and 80%	38%
E. fetida	11268-2, 2011	mean number of juveniles per replicate ≥ 30	98
		coefficient of variation of number of juveniles ≤ 30%	30%
E. crypticus	ISO 16387, 2004	adult mortality ≤ 20%	8%
		mean number of juveniles ≥ 25	1199
		coefficient of variation of number of juveniles ≤ 50%	43%
F. candida	ISO 11267, 2001	adult mortality ≤ 20%	12%
		mean number of juveniles ≥ 100	423
		coefficient of variation of number of juveniles ≤ 30%	28%

Table S.3: Concentrations of other cations and anions in the WW feed and percolates [mg/L] from four different soils (s1, s2, s3, s4) obtained at start, middle and end of the column experiment (sampling 1, 2, 3) measured in a 3 d composite sample (n=1).

Parameter	LOQ	Sampling	WW	percolates			
			feed	s1	s2	s3	s4
Ca	0.002	1	101	71	91	200	70
		2	100	100	79	91	103
		3	107	106	101	105	105
Na	0.2	1	128	58	66	85	50
		2	117	117	119	120	118
		3	124	126	129	129	128
K	0.2	1	31	6.1	0.7	11	48
		2	29	29	30	30	30
		3	31	31	32	31	32
Cl	0.1	1	132	105	107	100	106
		2	130	125	131	133	133
		3	142	134	143	147	145
NO_3^-	0.1	1	44	26	71	44	91
		2	39	34	34	37	37
		3	43	40	37	39	40
PO_4^{3-}	0.1	1	1.4	2.2	<LOQ	<LOQ	4.4
		2	<LOQ	1.1	<LOQ	<LOQ	0.9
		3	<LOQ	1.3	<LOQ	<LOQ	1.0
SO_4^{2-}	0.1	1	148	108	148	474	111
		2	140	105	134	136	136
		3	148	139	145	138	142
pH		1	7.4	7.4	5.9	6.5	7.8
		2	7.9	8.2	7.9	8.0	8.1
		3	7.8	8.0	7.8	8.0	8.1

Table S.4: Metal concentrations [µg/L] together with regulatory threshold values [µg/L] in WW feed and percolates from four different soils (s1, s2, s3, s4) obtained at start, middle and end of the column experiment (sampling 1, 2, 3) measured in a 3 d composite sample (n=1).

Metal	LOQ	Sampling	WW	percolates				AA-EQS	Drinking water threshold values[1]
			feed	s1	s2	s3	s4		
Al	100	1	<	<	160	100	<	na	200
		2	<	<	<	<	<		
		3	<	<	<	<	<		
As	5	1	<	<	<	<	18	50	10
		2	<	<	<	<	<		
		3	<	<	<	<	<		
Cu	2	1	60	190	30	20	40	1 - 28	2000
		2	10	20	5	<	20		
		3	10	10	<	<	5		
Ni	2	1	8	30	3	2	3	4	20
		2	<	10	<	<	<		
		3	2	5	<	<	<		
Zn	2	1	400	150	300	30	50	8 -125	
		2	280	30	30	10	10		
		3	720	40	10	<	7		

'<' for value smaller LOQ. AA-EQS: Environmental Quality Standards proposed according to the Water Framework Directive (WFD) for surface water bodies; for Ni from EU, 2013; for As, Cu, Zn from UK, 2008. Not depicted: Cd, Cr, Pb (always <2 µg/L) and Hg (always <5 µg/L).

EU 2013: Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy.

UK 2008: UK Technical Advisory Group on the Water Framework Directive, 2008. Proposal for Environmental Quality Standards for annex VIII substances. Final version SR1 – 2007; revised June 2008.

[1] EC 1998: European Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption.

Table S.5 A: Organic pollutants concentrations [ng/L] determined in WW feed and its percolates after passage through four soils (s1, s2, s3, s4) in three individual 24h composite samples during each of three sampling periods (1, 2, 3) or one composite sample of the three 24h samples per sampling period. Among the 95 analyzed organic substances, 45 were detected in the WW feed at all samplings and were considered here. (The remaining 47 substances are listed in Table S6). Substances listed in order of descending mean WW feed concentration.

Sampling period 1			Sample													
Substance	CAS-No	LOQ [ng/L]	WW feed	WW feed	WW feed	s1	s1	s1	s2	s2	s2	s3	s3	s3	s4	s4	s4
1H-Benzotriazole	95-14-7	50	14355	13399	12442	75	227	125	<LOQ	<LOQ	<LOQ	<LOQ	2442	5828	<LOQ	65	604
FAA	1672-58-8	50		6100			4750			2900			2300			4000	
Iopamidol	60166-93-0	50	4083	3283	3494	1827	2802	3638	1496	2865	3226	1384	2916	3778	1838	3405	4057
Iomeprol	78649-41-9	50	4789	4287	6943	1939	3065	8797	<LOQ	<LOQ	104	662	1736	4351	135	1047	2922
Acesulfame	33665-90-6	50	3173	3749	3640	1951	3407	4439	1466	3947	4079	1332	3132	4098	1567	3461	4488
Diatrizoat	117-96-4	200	3365	2695	2118	1449	2184	2430	1488	2581	2449	1279	2216	2812	1332	2344	2582
Diclofenac	15307-79-6	100	2949	2545	1891	<LOQ	79	670	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	148	990
DH-DH-CBZ	58955-93-4	20	2473	2375	1957	441	1667	2152	<LOQ	256	1218	15	76	584	242	1665	1643
CBZ	298-46-4	10	2605	2260	2193	8	11	116	<LOQ	<LOQ	<LOQ	11	<LOQ	<LOQ	<LOQ	22	345
O-Desmethyl-VLX	93413-62-8	20	2382	2234	2038	<LOQ	<LOQ	22	<LOQ	<LOQ	<LOQ	116	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
AAA	83-15-8	50		1850			1500			1100			800			1300	
Acyclovir-TP		20	2177	2258	2297	63	774	1550	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	232	<LOQ	151	788
TCPP	13674-84-5	200	1666	1339	1700	127	350	903	249	106	106	<LOQ	134	136	182	775	1383
Metoprolol	37350-58-6	5	68	1889	1815	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	49	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Iopromide	73334-07-3	50	887	893	1336	336	692	1348	<LOQ	51	139	176	438	930	75	342	706
BZP-4	4065-45-6	50	<LOQ	1781	1985	130	1053	1464	<LOQ	537	379	<LOQ	151	561	258	1640	1733
DHH-CBZ	29331-92-8	20	762	749	539	83	334	390	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	14	101	152
VLX	93413-69-5	10	749	671	641	<LOQ	<LOQ	46	<LOQ	<LOQ	<LOQ	72	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
SMX (P+TP)	723-46-6; 21312-10-7	20	1090	951	882	169	451	664	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	97	275	1190	1328
Primidone	125-33-7	20	561	494	563	177	429	552	<LOQ	234	437	56	146	379	139	463	607
PEMA	7206-76-0	100		350			300			300			200			300	
TnBP+TiBP	126-73-8; 126-71-6	20	403	251	441	1960	163	284	527	193	189	508	171	169	496	172	635
N,O-Didesmethyl-VLX		20	415	388	365	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Sotalol	3930-20-9	10	422	349	262	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
TBEP	78-51-3	20	55	60	241	<LOQ	38	35	183	25	<LOQ	247	<LOQ	<LOQ	189	<LOQ	237
3-OH-CBZ	68011-67-6	5	368	329	309	<LOQ	<LOQ	14	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	10	14
Acyclovir	59277-89-3	20	234	314	333	<LOQ	<LOQ	60	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
2-OH-CBZ	68011-66-5	5	261	229	288	<LOQ	6	27	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	12	33
AMDOPH	519-65-3	100		200			150			150			250			150	

Table S.5 A (continued)

Substance	CAS-No	LOQ [ng/L]	WW feed	WW feed	WW feed	s1	s1	s1	s2	s2	s2	s3	s3	s3	s4	s4	s4
AMPH	141330-37-2	100		200			<LOQ			<LOQ			<LOQ			200	
Bezafibrate	41859-67-0	20	154	239	223	10	65	127	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	6
Fluconazole	86386-73-4	5	231	204	193	<LOQ	42	117	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	26	16	141	191
Phenazon	60-80-0	50		200			150			<LOQ			<LOQ			150	
TCEP	115-96-8	20	235	170	139		160	145	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	123	143
Atenolol	29122-68-7	10	202	193	189	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Erythromycin	114-07-8	5	160	131	124	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Clarithromycin	81103-11-9	10	90	93	90	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
N-Desmethyl-VLX	149289-30-5	20	149	136	103	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Codeine	6059-47-8	20	88	85	96	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Trimethoprim	738-70-5	10	125	98	101	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Oxazepam	604-75-1	20	49	41	35	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Carbendazim	10605-21-7	10	65	54	47	11	<LOQ	<LOQ	17	<LOQ	<LOQ	9	<LOQ	<LOQ	8	<LOQ	<LOQ
Diuron	330-54-1	10	46	34	28	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Mecoprop	7085-19-0	20	36	29	20	13	24	20	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	21	<LOQ	15	18
Isoproturon	34123-59-6	5	18	15	14	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Table S.5 A (continued)

Sampling period 2			Sample													
Substance	**CAS-No**	**LOQ [ng/L]**	**WW feed**	**WW feed**	**WW feed**	**s1**	**s1**	**s1**	**s2**	**s2**	**s2**	**s3**	**s3**	**s3**	**s4**	**s4**	**s4**
1H-Benzotriazole	95-14-7	50	12619	14951	14633	11384	11553	11030	9758	11031	10883	9988	10423	11853	12144	12170	14473
FAA	1672-58-8	50		5750			800			350			1850			2400	
Iopamidol	60166-93-0	50	2514	2590	3083	3317	2498	2708	3629	2669	2742	3752	2712	2979	3592	2546	3124
Iomeprol	78649-41-9	50	2561	2775	6419	3221	1940	3197	3828	1934	3352	4695	2127	3521	3090	1749	4107
Acesulfame	33665-90-6	50	2315	2515	2242	2747	2324	2025	2425	2235	1781	2541	2250	2088	2713	2285	2338
Diatrizoat	117-96-4	200	2843	2756	2546	3156	2633	3034	4871	3261	2536	3915	3263	2430	3692	3081	2890
Diclofenac	15307-79-6	100	2462	2984	2782	2352	2152	2230	2018	2365	2117	2181	2026	2210	1816	1456	1802
DH-DH-CBZ	58955-93-4	20	2879	3419	3437	3192	3072	3002	3124	3304	3200	2962	2826	3128	2990	2959	3628
CBZ	298-46-4	10	1993	2320	2448	2547	2731	2681	1676	1924	1841	1293	1309	1463	3108	2832	3095
O-Desmethyl-VLX	93413-62-8	20	1511	1766	1809	1123	1207	1182	922	1063	1050	1128	1168	1273	1328	1344	1595
AAA	83-15-8	50		1900			400			250			750			900	
Acyclovir-TP		20	2068	2178	2379	1920	2123	2034	1699	2017	2002	1828	2059	2219	2039	2060	2650
TCPP	13674-84-5	200	1116	1681	1555	1361	1313	1584	1341	1474	1350	1360	1507	1640	1442	1440	1700
Metoprolol	37350-58-6	5	2189	2232	2318	531	561	549	270	317	311	65	69	73	626	629	745
Iopromide	73334-07-3	50	395	345	647	595	225	347	651	242	386	705	311	369	565	213	471
BZP-4	4065-45-6	50	2072	1278	1545	691	114	<LOQ	<LOQ	221	302	<LOQ	360	309	284	217	104
DHH-CBZ	29331-92-8	20	941	1101	845	337	294	243	594	594	447	37	28	24	319	212	340
VLX	93413-69-5	10	578	598	722	86	121	121	<LOQ	<LOQ	<LOQ	131	163	187	186	259	273
SMX (P+TP)	723-46-6; 21312-10-7	20	642	725	765	690	739	743	770	861	818	726	788	878	901	995	1078
Primidone	125-33-7	20	444	532	502	482	491	512	486	521	495	477	474	557	479	457	554
PEMA	7206-76-0	100		250			250			250			250			250	
TnBP+TiBP	126-73-8; 126-71-6	20	139	243	216	113	188	167	203	158	179	81	141	168	95	197	232
N,O-Didesmethyl-VLX		20	283	329	327	268	265	270	236	246	243	188	193	206	263	266	327
Sotalol	3930-20-9	10	342	385	382	312	365	379	333	366	339	182	206	221	318	327	489
TBEP	78-51-3	20	98	27	291	65	332	135	657	142	370	<LOQ	134	97	62	429	322
3-OH-CBZ	68011-67-6	5	251	308	308	237	226	226	172	193	186	142	137	151	175	152	159
Acyclovir	59277-89-3	20	252	240	268	147	134	136	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
2-OH-CBZ	68011-66-5	5	254	295	259	259	244	232	172	193	171	164	138	160	219	168	203
AMDOPH	519-65-3	100		200			200			200			200			200	

Table S.5 A (continued)

Substance	CAS-No	LOQ [ng/L]	WW feed	WW feed	WW feed	s1	s1	s1	s2	s2	s2	s3	s3	s3	s4	s4	s4
AMPH	141330-37-2	100		150			150			150			150			200	
Bezafibrate	41859-67-0	20	229	267	242	131	123	98	62	60	53	110	110	119	95	88	94
Fluconazole	86386-73-4	5	198	228	231	230	232	225	237	269	249	230	229	263	234	231	266
Phenazon	60-80-0	50		200			100			<LOQ			100			100	
TCEP	115-96-8	20	277	329	301	233	265	302	328	240	247	203	255	313	253	257	319
Atenolol	29122-68-7	10	153	183	183	16	22	<LOQ	16	19	20	<LOQ	<LOQ	<LOQ	19	22	20
Erythromycin	114-07-8	5	109	128	136	44	45	43	30	36	34	92	90	95	61	61	78
Clarithromycin	81103-11-9	10	126	150	137	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	51	52	58	<LOQ	<LOQ	<LOQ
N-Desmethyl-VLX	149289-30-5	20	90	113	103	73	71	71	<LOQ	<LOQ	22	<LOQ	<LOQ	<LOQ	89	77	106
Codeine	6059-47-8	20	106	120	108	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	22	23	30
Trimethoprim	738-70-5	10	105	116	130	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Oxazepam	604-75-1	20	66	119	80	51	114	66	11	<LOQ	31	24	<LOQ	12	58	108	<LOQ
Carbendazim	10605-21-7	10	36	40	40	26	26	25	<LOQ	11	12	<LOQ	<LOQ	<LOQ	22	24	27
Diuron	330-54-1	10	18	33	21	38	25	24	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	31	19	25
Mecoprop	7085-19-0	20	30	32	41	25	25	25	27	27	27	27	29	32	22	22	28
Isoproturon	34123-59-6	5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Table S.5 A (continued)

Sampling period 3			Sample													
Substance	**CAS-No**	**LOQ [ng/L]**	**WW feed**	**WW feed**	**WW feed**	**s1**	**s1**	**s1**	**s2**	**s2**	**s2**	**s3**	**s3**	**s3**	**s4**	**s4**	**s4**
1H-Benzotriazole	95-14-7	50	14932	13599	17723	13855	14124	19053	14931	13652	14102	14419	15164	13325	15178	16250	13187
FAA	1672-58-8	50		7850			100			100			100			950	
Iopamidol	60166-93-0	50	10345	8306	7812	14003	7967	8872	12521	13368	8433	10375	10480	6626	13308	7891	6995
Iomeprol	78649-41-9	50	6053	3633	5856	2257	1507	3678	3116	1860	2784	4493	2901	3255	3894	2171	3691
Acesulfame	33665-90-6	50	3295	3083	3832	2358	2496	3611	1568	1641	2127	1553	1618	1665	2263	2413	2462
Diatrizoat	117-96-4	200	4594	3443	3286	4594	3470	3925	5203	4431	4864	4450	2985	2769	4348	3445	2769
Diclofenac	15307-79-6	100	3089	2709	3193	2440	2402	2529	2274	1930	2044	2068	2077	2029	1843	1729	1765
DH-DH-CBZ	58955-93-4	20	1495	1518	1747	1853	1358	1884	1410	2173	1665	2349	1347	2878	1307	1830	3201
CBZ	298-46-4	10	2401	2276	2646	3732	3643	3872	2327	2388	2728	2277	2158	2195	3280	3407	2792
O-Desmethyl-VLX	93413-62-8	20	2260	2217	2668	2027	2003	2239	1816	1539	1813	2415	1940	1741	1852	2178	1770
AAA	83-15-8	50		2300			100			200			200			450	
Acyclovir-TP		20	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	1938	<LOQ	<LOQ	2067
TCPP	13674-84-5	200	976	1074	1157	303	395	628	468	409	470	328	487	430	629	558	658
Metoprolol	37350-58-6	5	117	806	157	231	246	278	289	255	261	191	189	167	352	353	236
Iopromide	73334-07-3	50	2396	2306	2014	891	615	1065	1714	1178	1134	1968	981	1209	1726	1143	970
BZP-4	4065-45-6	50	528	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	112
DHH-CBZ	29331-92-8	20	1413	1286	1455	155	147	195	207	181	186	<LOQ	<LOQ	21	374	388	281
VLX	93413-69-5	10	781	764	1006	432	458	488	324	320	320	511	470	375	665	712	510
SMX (P+TP)	723-46-6; 21312-10-7	20	649	639	758	701	702	881	900	861	754	886	814	1015	880	929	1117
Primidone	125-33-7	20	667	600	756	669	642	767	730	618	663	731	641	530	645	612	540
PEMA	7206-76-0	100		600			600			600			650			650	
TnBP+TiBP	126-73-8; 126-71-6	20	854	525	483	492	485	460	430	71	234	502	616	4374	442	414	3273
N,O-Didesmethyl-VLX		20	432	441	512	461	425	509	390	351	375	418	353	343	389	459	362
Sotalol	3930-20-9	10	410	379	470	360	364	434	367	349	354	258	263	167	400	338	246
TBEP	78-51-3	20	852	498	608	563	224	263	42	<LOQ	260	445	261	326	415	313	352
3-OH-CBZ	68011-67-6	5	264	227	250	331	263	301	198	209	238	301	211	204	129	132	130
Acyclovir	59277-89-3	20	277	293	277	57	102	74	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
2-OH-CBZ	68011-66-5	5	270	237	270	260	259	325	216	212	225	236	211	223	191	197	204
AMDOPH	519-65-3	100		350			300			350			350			350	
AMPH	141330-37-2	100		400			400			350			400			400	

Table S.5 A (continued)

Substance	CAS-No	LOQ [ng/L]	WW feed	WW feed	WW feed	s1	s1	s1	s2	s2	s2	s3	s3	s3	s4	s4	s4
Bezafibrate	41859-67-0	20	246	267	317	116	95	131	<LOQ	22	24	99	99	97	107	90	124
Fluconazole	86386-73-4	5	268	256	316	308	290	344	322	300	276	309	313	204	301	272	189
Phenazon	60-80-0	50		200			<LOQ			<LOQ			<LOQ			100	
TCEP	115-96-8	20	108	109	122	78	103	112	86	84	95	79	81	98	78	92	131
Atenolol	29122-68-7	10	127	190	192	18	20	26	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	26	32	31
Erythromycin	114-07-8	5	216	205	218	60	60	68	80	75	76	145	143	171	109	108	124
Clarithromycin	81103-11-9	10	168	148	182	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	112	109	119	<LOQ	<LOQ	<LOQ
N-Desmethyl-VLX	149289-30-5	20	142	144	171	124	129	152	92	82	83	69	70	68	140	154	149
Codeine	6059-47-8	20	146	141	182	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	28	25	<LOQ
Trimethoprim	738-70-5	10	122	129	132	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Oxazepam	604-75-1	20	93	89	110	71	67	81	27	27	26	<LOQ	<LOQ	<LOQ	80	80	69
Carbendazim	10605-21-7	10	48	40	49	42	42	50	25	28	25	10	<LOQ	<LOQ	36	34	37
Diuron	330-54-1	10	39	35	38	38	34	51	23	33	22	11	<LOQ	<LOQ	33	39	31
Mecoprop	7085-19-0	20	24	37	35	<LOQ	25	29	<LOQ	<LOQ	24	<LOQ	<LOQ	26	<LOQ	<LOQ	27
Isoproturon	34123-59-6	5	22	19	20	21	22	24	22	23	20	24	25	22	22	20	17

Table S.5 B: Organic pollutants concentrations in WW feed (mean of nine 24 h-composite samples) as well as their removal by soil passage averaged over four soils and the sampling periods 2 and 3 (sampling 1 omitted in order to consider only steady-state removal).

Substance	CAS No	LOQ	**Mean WW feed conc., (averaged over samplings 1, 2, 3) [µg/L]**	Mean removal, (samplings 2 and 3 [%])	Stdev [%]
1H-Benzotriazole	95-14-7	0.050	**14**	11	11
FAA	1672-58-8	0.050	**6.6**	86	15
Iopamidol	60166-93-0	0.100	**5.1**	-16	7
Iomeprol	78649-41-9	0.050	**4.8**	33	14
Acesulfame	33665-90-6	0.100	**3.1**	20	22
Diatrizoate	117-96-4	0.200	**3.1**	-14	12
Diclofenac	15307-79-6	0.020	**2.7**	28	9
DH-DH-CBZ	58955-93-4	0.010	**2.4**	-2	8
CBZ	298-46-4	0.005	**2.3**	-8	31
O-Desmethyl-VLX	93413-62-8	0.010	**2.4**	2.1	11
AAA	83-15-8	0.050	**2.0**	80	15
Acyclovir-TP		0.020	**1.5**	>3.5	5.7
TCPP	13674-84-5	0.100	**1.4**	28	31
Metoprolol	37350-58-6	0.005	**1.3**	54	33
Iopromide	73334-07-3	0.050	**1.2**	26	21
BZP-4	4065-45-6	0.100	**1.0**	>80	6.8
DHH-CBZ	29331-92-8	0.010	**1.0**	>78	18
VLX	93413-69-5	0.020	**0.72**	>61	25
SMX (P+TP)	723-46-6; 21312-10-7	0.020	**0.79**	-20	12
Primidone	125-33-7	0.020	**0.57**	0	3
PEMA	7206-76-0	0.100	**0.40**	-2	4
TnBP+TiBP	126-73-8; 126-71-6	0.020	**0.40**	25	17
N,O-Didesmethyl-VLX		0.020	**0.46**	0.4	11
Sotalol	3930-20-9	0.010	**0.38**	16	17
TBEP	78-51-3	0.020	**0.30**	-5	91
3-OH-CBZ	68011-67-6	0.005	**0.29**	24	25
Acyclovir	59277-89-3	0.020	**0.28**	>84	17
2-OH-CBZ	68011-66-5	0.005	**0.26**	20	16
AMDOPH	519-65-3	0.100	**0.25**	2	5
AMPH	141330-37-2	0.100	**0.25**	-3	13
Bezafibrate	41859-67-0	0.005	**0.24**	66	14
Fluconazole	86386-73-4	0.005	**0.24**	-9	4
Phenazon	60-80-0	0.050	**0.20**	>63	13
TCEP	115-96-8	0.100	**0.20**	17	8
Atenolol	29122-68-7	0.020	**0.18**	>90	4
Erythromycin	114-07-8	0.020	**0.16**	52	19
Clarithromycin	81103-11-9	0.005	**0.13**	>82	23

Table S.5 B (continued)

Substance	CAS No	LOQ	**Mean WW feed conc., (averaged over samplings 1, 2, 3) [µg/L]**	Mean removal, (samplings 2 and 3 [%])	Stdev [%]
N-Desmethyl-VLX	149289-30-5	0.020	**0.15**	>39	31
Codeine	6059-47-8	0.020	**0.12**	>84	3
Trimethoprim	738-70-5	0.010	**0.12**	>92	0
Oxazepam	604-75-1	0.020	**0.076**	47	36
Carbendazim	10605-21-7	0.005	**0.047**	>46	28
Diuron	330-54-1	0.005	**0.032**	>24	36
Mecoprop	7085-19-0	0.010	**0.032**	>30	9
Isoproturon	34123-59-6	0.010	**0.020**	>-4	7

In case values in the percolates were <LOQ, they were replaced by LOQ thus estimating minimum removal . Among the 95 analyzed organic substances, 45 were detected in the WW feed at all samplings and were considered here. (The remaining 47 substances are listed in Table S6). Substances listed in order of descending mean WW feed concentration. Stdev = standard deviation.

Abbreviations:

CBZ	Carbamazepine
DH-DH-CBZ	10,11-Dihydro-10,11-dihydroxy-CBZ
DHH-CBZ	10,11-Dihydro-10-hydroxy-CBZ
VLX	Venlafaxine
TCC	Triclocarban
TCS	Triclosan
SMX	Sulfamethoxazole (parent compound) and acetyl-sulfamethoxazole (TP)
TCPP	Tris(2-chlorisopropyl)phosphate
TCEP	Tris(2-chlorethyl)phosphate
TBEP	Tris(2-butoxyethyl)phosphate
TnBP	Tris(n-butyl)phosphate
TiBP	Triisobutylphosphate
FAA	4-formylaminoantipyrine
AAA	4-acetylaminoantipyrine
BZP-4	Benzophenone-4
PEMA	2-ethyl-2-phenyl-malonamide
AMDOPH	1-Acetyl-1-methyl-2-dimethyl-oxamoyl-2-phenylhydrazid

Table S.6: Percentage of 45 organic pollutants in percolates falling into the classes of poor (≤40%), moderate (41%-70%) and high (71%-100%) removal, after passage of WW feed through four different soils (s1, s2, s3, s4) sampled at three sampling periods (start, middle, end of experiment). The same substances were considered as listed in Table S5.

		Poor	Moderate	High	Moderate + high		Average	
		≤ 40%	41% - 70%	71% - 100%	(No.)	in %	No.	in %
Sampling 1	**s1**	16	6	23	29	60		
	s2	7	4	34	38	79		
	s3	6	8	31	39	81		
	s4	14	7	24	31	65	34	71
Sampling 2	**s1**	31	5	9	14	29		
	s2	26	2	17	19	40		
	s3	24	9	12	21	44		
	s4	30	8	7	15	31	17	36
Sampling 3	**s1**	28	6	11	17	35		
	s2	22	10	13	23	48		
	s3	25	8	12	20	42		
	s4	27	9	9	18	38	20	41

Table S.7: List of organic pollutants measured in WW feed but not detected above their LOQ.

Substance	LOQ [ng/L]	Substance	LOQ [ng/L]
Chlorophene	100	Simazine	50
TCC	10	Lenacil	50
TCS	100	Metalaxyl	50
PFOA	20	Metazachlor	50
PFOS	20	Metolachlor	100
Propyphenazone	50	Quinoxyfen	50
DMAA	50	Terbutylazine	50
Phenacetin	50	Bromoxynil	100
Methylphenacetin	50	2,4,5-T	100
Meprobamat	150	Bentazone	100
Pyrithyldione	50	Dichlorprop	100
Sulfamerazine	50	SPS	100
Tolbutamide	50	2.4-D	100
Phenytoine	500	MCPA	100
Alachlor	50	Clofibric acid	100
Atrazine	50	N-ASO	100
Boscalid	200		
Bromacil	100		
Chloridazone	50		
Chlorfenvinphos	150		
Chlortolurone	50		
Desethylterbutylazine	50		
Desethylatrazine	50		
Desphenylchloridazone	150		
Desphenylmethylchloridazone	50		
Desisopropylatrazin	50		
Dichlorbenzamide	50		
Ethofumesat	200		
Fenuron	100		
Metamitrone	50		
Metribuzine	50		

Table S.8: Effect data for *Daphnia*, green algae and *Lemna* used for the calculation of sum toxic units (sum TUs) for organic pollutants in WW feed and percolate samples.

Effect data used for *Daphnia*						
substance	***CAS***	***Use***	***EC50 [µg/L]***	***details***	***source***	***details source***
Carbamazepine	298-46-4	Anticonvulsant	111000	D. magna immobilisation 48h	A	155862 Han,G.H., H.G. Hur, and S.D. Kim, 2006
Clarithromycin	81103-11-9	Antibacterial agent	8160	Ceriodaphnia dubia reproduction, 48h	A	102321 Isidori,M., M. Lavorgna, A. Nardelli, L. Pascarella, and A. Parrella, 2005
Erythromycin	114-07-8	Antibacterial agent	24000	Daphnia longispina, 48h	A	154108 El-Bassat,R.A., H.E. Touliabah, G.I. Harisa, and F.A.Q. Sayegh, 2011
Trimethoprim	738-70-5	Antibacterial agent	92000	D. magna immobilisation 48h	A	119413 Park,S., and K. Choi, 2008
Sulfamethoxazole	723-46-6	Antibacterial agent	123100	D. magna immobilisation 48h	A	119413 Park,S., and K. Choi, 2008
Atenolol	29122-68-7	Beta-Blockers	750000	D. magna immobilisation 48h	A	160488 De Andres,F., G. Castaneda, and A. Rios, 2009
Metoprolol	51384-51-1	Beta-Blockers	> 100000	D. magna immobilisation 48h	A	153670 Cleuvers,M., 2003
Climbazole	38083-17-9	Fungicide	15990	D. magna, 48h	B	ECT
Fluconazole	86386-73-4	Fungicide	166400	D. magna reproduction, 21d	B	ECT
Propiconazole	60207-90-1	Fungicide	3200	D. magna, 48h	A	344 U.S. Environmental Protection Agency, and Office of Pesticide Programs , 2013
Diuron	330-54-1	Herbicide	2000	D. magna, 48h	A	102051 Malato,S., J. Caceres, A.R. Fernandez-Alba, L. Piedra, M.D. Hernando, A. Aguera, and J. Vial, 2003
Isoproturon	34123-59-6	Herbicide	> 1000	D. magna, 48h	A	8479 Traunspurger,W., H. Schafer, and A. Remde, 1996
Terbutryn	886-50-0	Herbicide	7100	D. magna, 48h	A	13154 Marchini,S., L. Passerini, D. Cesareo, and M.L. Tosato, 1988
Carbendazim	10605-21-7	Fungicide	156	D. magna, 48h	A	103653 Ferreira,A.L.G., S. Loureiro, and A.M.V.M. Soares, 2008
Bezafibrate	41859-67-0	Lipid regulator	30300	D. magna, 48h	A	155862 Han,G.H., H.G. Hur, and S.D. Kim, 2006
Diclofenac	15307-86-5	Analgesic agent	10000	D. magna, 48h	A	151753 Cleuvers,M., 2004
Mecoprop	93-65-2	Herbicide	> 10000	D. magna, 48h	A	159999 Matsumoto,K.I., M. Hosokawa, K. Kuroda, and G. Endo, 2009
Amphetamine sulfate	300-62-9	Stimulating agent	22174	D. magna, 24h	A	16756 Lilius,H., B. Isomaa, and T. Holmstrom, 1994
Atrazine	93616-39-8	Herbicide	35500	D. magna, 48h	A	108323 Palma,P., V.L. Palma, R.M. Fernandes, A.M.V.M. Soares, and I.R. Barbosa, 2008
TCCP	14609-54-2	Flame retardant	13850	D. magna, 48h	B	ECT

Table S.8 (continued)

Effect data used for green algae						
substance	***CAS***	***Use***	***EC50 [µg/L]***	***details***	***source***	***details source***
Carbamazepine	298-46-4	Anticonvulsant	89120	S. vacuolatus, 72h	A	157681 Zhang,W., M. Zhang, K. Lin, W. Sun, B. Xiong, M. Guo, X. Cui, and R. Fu, 2012
Clarithromycin	81103-11-9	Antibacterial agent	2	P. subcapitata, 72h	A	102321 Isidori,M., M. Lavorgna, A. Nardelli, L. Pascarella, and A. Parrella, 2005
Erythromycin	114-07-8	Antibacterial agent	20	P. subcapitata, 72h	A	102321 Isidori,M., M. Lavorgna, A. Nardelli, L. Pascarella, and A. Parrella, 2005
Trimethoprim	738-70-5	Antibacterial agent	80300	P. subcapitata, 72h	A	76739 Eguchi,K., H. Nagase, M. Ozawa, Y.S. Endoh, K. Goto, K. Hirata, K. Miyamoto, and H. Yoshimura, 2004
Sulfamethoxazole	723-46-6	Antibacterial agent	520	P. subcapitata, 72h	A	102321 Isidori,M., M. Lavorgna, A. Nardelli, L. Pascarella, and A. Parrella, 2005
Atenolol	29122-68-7	Beta-Blockers	143000	P. subcapitata, 72h	A	160488 De Andres,F., G. Castaneda, and A. Rios, 2009
Metoprolol	51384-51-1	Beta-Blockers	14390	P. subcapitata, 72h	B	ECT
Climbazole	38083-17-9	Fungicide	214	P. subcapitata, 72h	B	ECT
Fluconazole	86386-73-4	Fungicide	18500	P. subcapitata, 72h, RF	B	ECT
Propiconazole	60207-90-1	Fungicide	390	P. subcapitata, 72h	A	119412 Ochoa-Acuna,H.G., W. Bialkowski, G. Yale, and L. Hahn, 2009
Diuron	330-54-1	Herbicide	11	P. subcapitata, 72h	A	102060 Fai,P.B., A. Grant, and B. Reid, 2007
Isoproturon	34123-59-6	Herbicide	142	P. subcapitata, 72h	A	103498 Arzul,G., F. Quiniou, and C. Carrie, 2006
Terbutryn	886-50-0	Herbicide	2	P. subcapitata, 72h	A	56747 Okamura,H., I. Aoyama, D. Liu, R.J. Maguire, G.J. Pacepavicius, and Y.L. Lau, 2000
Carbendazim	10605-21-7	Fungicide	1400	Chlorella pyrenoidosa, 48h	A	5483 Canton,J.H., 1976
Bezafibrate	41859-67-0	Lipid regulator	60000	P. subcapitata, 72h	A	156160 Isidori,M., A. Nardelli, L. Pascarella, M. Rubino, and A. Parrella, 2007
Diclofenac	15307-86-5	Analgesic agent	64800	P. subcapitata, 72h	A	155069 Quinn,B., W. Schmidt, K. O'Rourke, and R. Hernan, 2011
Mecoprop	93-65-2	Herbicide	102660	P. subcapitata, 72h	A	13695 Kirby,M.F., and D.A. Sheahan, 1994
Amphetamine sulfate	300-62-9	Stimulating agent	na			
Atrazine	93616-39-8	Herbicide	2	P. subcapitata, 72h	A	98204 Sbrilli,G., B. Bimbi, F. Cioni, L. Pagliai, F. Luchi, and E. Lanciotti, 2005
TCCP	14609-54-2	Flame retardant	31600	P. subcapitata, 72h	B	ECT

Table S.8 (continued)

Effect data used for *Lemna*						
substance	***CAS***	***Use***	***EC50 [µg/L]***	***details***	***source***	***details source***
Erythromycin	114-07-8	Antibacterial agent	5620	L. minor, 7d	C	
Diuron	330-54-1	Herbicide	18	L. gibba, 7d	D	
Mecoprop	93-65-2	Herbicide	5147	L. minor, 7d	A	13695 Kirby,M.F., and D.A. Sheahan, 1994
Atrazin	93616-39-8	Herbicide	56	L. minor, 10d	A	13695 Kirby,M.F., and D.A. Sheahan, 1994
Sulfamethoxazole	723-46-6	Antibacterial agent		L. gibba, 7d	A	73383 Brain,R.A., D.J. Johnson, S.M. Richards, H. Sanderson, P.K. Sibley, and K.R. Solomon, 2004
Climbazole	38083-17-9	Fungicide	19	L. minor, 7d	B	

Source: A = data bank ECOTOX (http://cfpub.epa.gov/ecotox/), May 2014; B = own data (ECT GmbH); C = wikipharma (http://www.wikipharma.org/api_data.asp); D = TOXNET (http://toxnet.nlm.nih.gov).

Table S.9: Sums of toxic units (sum TUs) for green algae and Lemna considering 19 (for algae) and 6 (for Lemna) organic pollutants in WW feed and percolate samples and their effective concentrations (EC50, as described in Table S7).

Sampling	Sample	Sum TUs green algae	Sum TUs Lemna
1	WW feed	0.080	0.272
	s1	0.018	0.192
	s2	0.018	0.218
	s3	0.018	0.210
	s4	0.018	0.093
2	WW feed	0.103	0.089
	s1	0.076	0.057
	s2	0.019	0.039
	s3	0.050	0.043
	s4	0.174	0.053
3	WW feed	0.125	0.096
	s1	0.086	0.066
	s2	0.115	0.091
	s3	0.164	0.081
	s4	0.099	0.064

Table S.10: Organic pollutants detected in four different soils (s1, s2, s3, s4) exclusively after column percolation with treated wastewater (WW) for 77 days but not detected in the untreated soils (CON).

(A) Measured soil concentrations (n=3 replicate measurements) in µg/kg soil dry weight with standard deviation (std).

soil			s1			s2			s3			s4		
Substance	CAS No	LOQ [µg/kg]	mean	±	std	mean	±	std	mean	±	std	mean	±	std
CBZ	298-46-4	1.0	18	±	0.7	76	±	1.1	151	±	5.9	12	±	0.7
DH-DH-CBZ		1.0	3.1	±	0.2	9.8	±	0.3	24	±	0.4	2.9	±	0.1
2-OH-CBZ		1.0	1.7	±	0.1	6.0	±	0.3	13	±	0.2	<LOQ	±	<LOQ
3-OH-CBZ		1.0	2.1	±	0.0	7.2	±	0.5	17	±	0.2	<LOQ	±	<LOQ
Oxazepam	604-75-1	2.0	<LOQ	±	<LOQ	5.6	±	0.4	8.8	±	0.8	<LOQ	±	<LOQ
Tramadol	36282-47-0	5.0	15	±	0.7	43	±	4.1	70	±	16.2	7.4	±	0.7
VLX	93413-69-5	2.0	13	±	2.1	22	±	4.4	37	±	11.7	6.3	±	0.4
N-Desmethyl-VLX	n.a.	2.0	<LOQ	±	<LOQ	3.2	±	0.5	5.2	±	0.2	<LOQ	±	<LOQ
O-Desmethyl-VLX	n.a.	5.0	18	±	2.6	27	±	4.0	45	±	6.4	8.4	±	0.2
N,O-Didesmethyl-VLX	n.a.	2.0	2.4	±	0.1	6.3	±	0.7	9.5	±	1.2	<LOQ	±	<LOQ
Atenolol	29122-68-7	2.0	<LOQ	±	<LOQ	<LOQ	±	<LOQ	3.0	±	0.6	<LOQ	±	<LOQ
Metoprolol	37350-58-6	0.5	6.5	±	0.0	16	±	1.2	35	±	6.6	6.0	±	0.6
Clarithromycin	81103-11-9	1.0	3.3	±	0.1	3.4	±	0.3	10	±	0.8	3.3	±	0.1
Erythromycin	114-07-8	2.0	<LOQ	±	<LOQ	3.2	±	2.3	4.9	±	0.2	<LOQ	±	<LOQ
SMX	723-46-6	1.0	2.6	±	0.1	5.5	±	0.1	13.2	±	0.3	1.8	±	0.1
Trimethoprime	738-70-5	1.0	3.0	±	0.1	5.0	±	0.3	7.6	±	1.0	1.9	±	0.2
1H-Benzotriazole	95-14-7	5.0	194	±	5.6	229	±	9.8	431	±	7.6	102	±	3.8
Diclofenac	15307-79-6	2.0	9.0	±	0.4	32	±	4.9	55	±	3.4	3.0	±	0.4
Carbendazim	10605-21-7	0.5	1.5	±	0.1	2.8	±	0.1	5.2	±	0.2	1.2	±	0.3
Fluconazole	86386-73-4	0.5	0.56	±	0.0	1.6	±	0.2	2.2	±	0.1	<LOQ	±	<LOQ
Diuron	330-54-1	1.0	1.2	±	0.1	2.9	±	0.2	5.1	±	0.1	1.2	±	0.1

(B) Concentrations normalised to TOC (soil concentration divided by TOC fraction of the respective soil), in µg/kg TOC, with standard deviation (std).

soil		s1			s2			s3			s4		
Substance	CAS No	mean	±	std	mean	±	std	mean	±	std	mean	±	std
CBZ	298-46-4	1081	±	38	1315	±	19	1620	±	63	1131	±	60
DH-DH-CBZ		185	±	12	169	±	5	258	±	5	267	±	9
2-OH-CBZ		97	±	5	103	±	6	143	±	2	<LOQ	±	<LOQ
3-OH-CBZ		123	±	1	124	±	8	179	±	2	<LOQ	±	<LOQ
Oxazepam	604-75-1	<LOQ	±	<LOQ	97	±	7	94	±	9	<LOQ	±	<LOQ
Tramadol	36282-47-0	903	±	42	739	±	71	749	±	174	676	±	63
VLX	93413-69-5	792	±	124	383	±	77	403	±	125	577	±	38
N-Desmethyl-VLX	n.a.	<LOQ	±	<LOQ	56	±	8	56	±	2	<LOQ	±	<LOQ
O-Desmethyl-VLX	n.a.	1035	±	151	469	±	69	479	±	69	760	±	22
N,O-Didesmethyl-VLX	n.a.	140	±	4	108	±	12	102	±	13	<LOQ	±	<LOQ
Atenolol	29122-68-7	<LOQ	±	<LOQ	<LOQ	±	<LOQ	32	±	6	<LOQ	±	<LOQ
Metoprolol	37350-58-6	382	±	1	275	±	21	372	±	71	549	±	59
Clarithromycin	81103-11-9	192	±	9	59	±	5	107	±	9	299	±	9
Erythromycin	114-07-8	<LOQ	±	<LOQ	55	±	40	53	±	2	<LOQ	±	<LOQ
SMX	723-46-6	156	±	5	95	±	1	141	±	3	161	±	13
Trimethoprime	738-70-5	174	±	8	87	±	5	82	±	11	169	±	15
1H-Benzotriazole	95-14-7	11384	±	329	3942	±	169	4640	±	81	9249	±	349
Diclofenac	15307-79-6	529	±	24	554	±	85	596	±	37	274	±	38
Carbendazim	10605-21-7	91	±	5	49	±	2	55	±	2	113	±	23
Fluconazole	86386-73-4	33	±	1	27	±	3	24	±	1	<LOQ	±	<LOQ
Diuron	330-54-1	70	±	6	50	±	3	55	±	1	111	±	6

TOC in WW percolated soils was 1.7%, 5.8%, 9.3% and 1.1%, respectively in s1, s2, s3 and s4.

Abbreviations:

CBZ	Carbamazepine
DH-DH-CBZ	10,11-Dihydro-10,11-dihydroxy-CBZ
VLX	Venlafaxine
SMX	**Sulfamethoxazole**

Table S.11: Classical organic pollutants monitored in four different soils (s1, s2, s3, s4) after percolation with treated wastewater (WW) and untreated (CON). Concentrations measured in one homogenised sample per soil (n=3 replicate measurements for QACs, n=1 for other groups) in µg/kg soil dry weight.

Table S.11: Classical organic pollutants monitored in four different soils (s1, s2, s3, s4) after percolation with treated wastewater (WW) and untreated (CON). Concentrations measured in one homogenised sample per soil (n=3 replicate measurements for QACs, n=1 for other groups) in µg/kg soil dry weight.

			Untreated (CON)				Percolated (WW)			
Group	**Substance**	**LOQ**	**s1**	**s2**	**s3**	**s4**	**s1**	**s2**	**s3**	**s4**
QACs	Benzethonium chloride	2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Benzyldimethyldodecyl ammoniumchloride	2	15	<LOQ	<LOQ	<LOQ	9	<LOQ	<LOQ	<LOQ
	Benzyldimethylhexadecyl ammoniumchloride	8	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Benzyldimethyloctadecyl ammoniumchloride	15	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Benzyldimethyltetradecyl ammoniumchloride	2	3.8	<LOQ	<LOQ	<LOQ	3.6	<LOQ	<LOQ	<LOQ
	Didecyldimethyl ammoniumchloride	8	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Dihexadecyldimethyl ammoniumchloride	15	297	<LOQ	<LOQ	<LOQ	260	<LOQ	<LOQ	27
	Ditetradecyldimethyl ammoniumchloride	2	2.3	<LOQ	<LOQ	<LOQ	4.8	<LOQ	<LOQ	<LOQ
	Hexadecyltrimethyl ammoniumchloride	3	5.4	<LOQ	<LOQ	<LOQ	4.1	<LOQ	<LOQ	<LOQ
PAHs	Naphthalene		54	22	18	17	53	34	27	23
	Acenaphthylene		33	22	6.4	9.3	16	11	6.2	15
	Acenaphthene		9.9	5.6	1.8	1.7	7.4	6.3	2.2	5.1
	Fluorene		32	17	8.9	7.2	26	15	11	8
	Phenanthrene		291	163	23	68	237	140	23	68
	Anthracene		70	21	2.7	18	47	18	3.4	22
	Fluoranthene		1830	1093	88	789	1547	1011	76	648
	Pyrene		878	445	37	301	732	490	37	315
	Cyclopenta(c,d)pyrene		14	6.3	1	3.1	10	7.4	1.2	3
	Benz(a)anthracene		958	499	35	464	983	639	31	449
	Triphenylene		67	38	3.1	27	75	50	3.7	27
	Chrysene		206	114	8.8	81	186	130	9	86
	Benzo(b)fluoranthene		320	200	16	126	312	214	16	132
	Benzo(k)fluoranthene		128	67	5.3	48	118	86	3.3	56
	Benzo(e)pyrene		44	25	2.3	17	60	42	3.2	25
	Benzo(a)pyrene		142	75	5.3	62	121	82	4.9	38
	Dibenzo(a,c)anthracene		197	93	3.4	59	119	68	1.6	38
	Indeno(1,2,3-cd)pyrene		321	190	19	107	253	152	14	100

Table S.11 (continued)

			Untreated (CON)				Percolated (WW)			
Group	**Substance**	**LOQ**	**s1**	**s2**	**s3**	**s4**	**s1**	**s2**	**s3**	**s4**
	Dibenzo(a,h)anthracene		41	27	5.5	16	32	25	5	15
	Benzo(g,h,i)perylene		345	185	18	108	272	157	15	98
	Benzo-b-chrysene		62	34	8.1	23	41	29	1.7	15
	Benzo(j)fluoranthene		122	68	5.5	51	122	83	5.4	55
Tin organics	MBT		25	1.1	1	0.8	19	<LOQ	<LOQ	<LOQ
	DBT		4.4	1.7	1.9	0.6	3.3	0.6	1	<LOQ
	TBT		1.8	0.4	0.8	0.6	1.8	0.5	0.9	0.5
	MOT		0.9	0.6	1.3	1.3	0.8	<LOQ	0.7	<LOQ
	TTBT		<LOQ	0.8	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	DOT		<LOQ	2	4.5	4.2	<LOQ	0.7	0.5	0.7
	TCxT		<LOQ	<LOQ	0.5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	TPhT		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
PCBs	PCB28		82	0	0.1	0	69	0.2	0.1	0
	PCB31		69	0.1	0.1	0	59	0.2	0.1	0
	PCB49		57	0.1	0.1	0	57	0.2	0.2	0.1
	PCB52		69	0.1	0.2	0	66	0.3	0.2	0.1
	PCB101		56	0.2	0.1	0.1	55	0.4	0.1	0.2
	PCB105		17	0.1	0.1	0	16	0.1	0	0
	PCB118		38	0.1	0.1	0.1	38	0.2	0.1	0.1
	PCB138		25	0.2	0	0.3	23	0.2	0	0.3
	PCB149		33	1	0.2	0.7	33	1.1	0.2	0.8
	PCB153		38	0.9	0.2	0.8	38	1	0.1	0.9
	PCB156		5.2	0.1	0	0.1	5.1	0.1	0	0.1
	PCB170		10	0.4	<LOQ	0.5	9.3	0.4	0.1	0.5
	PCB180		16	0.5	0.2	0.7	15	0.6	0.1	0.7
	PCB194		3.3	0.2	0.1	0.2	3	0.1	0.1	0.2
	PCB209		0	0	<LOQ	0	0	0	<LOQ	0

Table S.11 (continued)

			Untreated (CON)				Percolated (WW)			
Group	**Substance**	**LOQ**	**s1**	**s2**	**s3**	**s4**	**s1**	**s2**	**s3**	**s4**
NVHHs	1,2,3TriCB		0.2	0	0	0	0.3	0	0	0
	1,2,4-TriCB		2.8	<LOQ	<LOQ	0.3	3.3	<LOQ	0.6	<LOQ
	1,3,5-TriCB		0	<LOQ	<LOQ	0	0.1	0.1	<LOQ	<LOQ
	TeCB		0.1	0	<LOQ	0	0.1	0	0	0
	PeCB		0.3	0.1	<LOQ	0	0.5	0.2	0	0
	HCB		2.2	0.4	0.1	0	2.1	0.3	0.1	0
	A-HCH		0.2	0.2	0.3	0.1	0.3	0.2	0.1	0.1
	B-HCH		0.4	0.4	0.6	0.2	0.4	0.1	0.1	0.1
	G-HCH		0.5	0.4	0.6	0.2	0.4	0.2	0.1	0.1
	opDDT		3.7	25	0.1	4	3.6	29	0	4.1
	ppDDT		57	237	0.3	40	56	325	0.5	54
	opDDD		12	4.8	0	0.1	13	4.8	0.1	0.1
	ppDDD		20	18	0.2	0.5	21	19	0.4	0.4
	opDDE		0.4	1.1	<LOQ	0.4	0.4	1.3	<LOQ	0.3
	ppDDE		6.2	57	0.2	53	6.1	56	0.2	49
	Aldr		128	196	103	78	72	<LOQ	147	<LOQ
	HCBD		10	18	14	2	11	11	7	7

Abbreviations:

QACs	Quaternary ammonia compounds
PAHs	Polyaromatic hydrocarbons
PCBs	Polychlorinated diphenyls
NVHHs	non-volatile halogenated hydrocarbons
MBT	Monobutyltin
DBT	Dibutyltin
TBT	Tributyltin
MOT	Monooctyltin
TTBT	Tetrabutyltin
DOT	Dioctyltin
TCxT	Tricyclohexyltin
TPhT	Triphenyltin
TriCB	Trichlorobenzene
TeCB	Tetrachlorobenzene
PeCB	Pentachlorobenzene
HCB	Hexachlorobenzene
HCH	Alpha-Hexachlorocyclohexane
DDT	Dichlorodiphenyltrichloroethane
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethene
HeCL	Heptachlor
Aldr	Aldrin
PClAn	Pentachloroanisole
HCBD	Hexachlorobutadiene