EMERGING CONTAMINANTS AND THEIR (IMMUNOTOXIC) EFFECTS ON AQUATIC ORGANISMS

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Abbreviations

ACV	Acyclovir
AOPs	Advanced oxidation processes
BMBF	Federal Ministry of Education and Research (German: Bundesministerium für Bildung und Forschung)
BOD	Biological oxygen demand
CA	Concentration addition
C-ACV	Carboxy-acyclovir
COD	Chemical oxygen demand
COFA	N-(4-carbamoyl-2-imino-5-oxoimidazolidine)formamido-N-methoxy-acetic acid
CsA	Cyclosporine A
DDE	Dichlorodiphenyldichloroethylene
ERA	Environmental risk assessment
GAC	Granulated activated carbon
MBR	Membrane bioreactor
OgewV	Oberflächengewässerverordnung
PAC	Powdered activated carbon
PBT	Persistent, bioaccumulative and toxic substances
PCBs	Polychlorinated biphenyls
PPCPs	Pharmaceuticals and personal care products
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
TOC	Total organic carbon
TPs	Transformation products
TSS	Total suspended solids
vPvB	Very persistent and very bioaccumulative
WFD	Water Framework Directive
WHG	Wasserhaushaltsgesetz
WWTP	Wastewater treatment plant

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Summary

To date, chemicals are used ubiquitous in everyday life and an increasing consumption of pharmaceuticals and personal care products and industrial chemicals results in an increased water pollution. Conventional wastewater treatment plants are not able to completely remove the variety of (polar) organic compounds from today's wastewater and thus serve as constant key point sources for the unintentional release of (micro-)pollutants into the aquatic environment. Anthropogenic micropollutants are detectable in very low concentrations in almost every aquatic compartment and may cause adverse effects on aquatic organisms. Considering the current situation of water pollution and to enhance water quality with regard to environmental and human health, the implementation of advanced wastewater treatment technologies, such as ozonation and activated carbon filtration was extensively discussed and investigated in recent years. Yet, besides their advantages regarding the efficient removal of a variety of recalcitrant, organic compounds as well as pathogens from the wastewater, it is known that especially the treatment with ozone may lead to the formation of largely unknown ozonation by-products with often unknown toxicity and unknown threats to human and the environment. To address these topics the joint research project *TransRisk* aimed at the "characterization, communication and minimization of risks originating from emerging contaminants and pathogens in the water cycle". Within this research project the present thesis focuses on the ecotoxicological investigation of emerging waterborne contaminants, including their potential transformation products (TPs). Additionally, focus was laid on the investigation of combined effects of anthropogenic contaminants and pathogens with effects especially on aquatic invertebrate organisms.

The potential ecotoxicological effects of the antiviral drug acyclovir and two of its structurally identified TPs, were investigated on three aquatic organisms (*Raphidocelis subcapitata, Daphnia magna* and embryos of *Danio rerio*). While the parent compound acyclovir caused no acute toxicity up to a tested concentration of 100 mg/l on any of the investigated organisms, both TPs were shown to exhibit an increased aquatic toxicity. Carboxy-acyclovir, the biodegradation product of

acyclovir, significantly reduced reproduction of *D. magna* by 40% at 102 mg/l, and the ozonation product COFA significantly inhibited growth of green algae *R. subcapitata* (EC_{10} = 14.1 mg/l). In the present case, advanced wastewater treatment was shown to lead to the formation of TPs, that reveal a higher toxicity towards investigated organisms, than the parent compound. Results highlight the necessity of further research related to the topic of identification and characterization of TPs, formed during advanced wastewater treatment processes.

To investigate the potential reduction or enhancement of toxic effects of nine differently treated wastewater effluents, selected bioassays with *Daphnia magna*, *Lumbriculus variegatus* and *Lemna minor* were conducted in flow-through test systems on a pilot treatment plant. The different treatment processes included ozonation of conventional biological treatment, with subsequent filtration processes as well as membrane bioreactor treatment in combination with ozonation. While exposure to the conventionally treated wastewater did not result in significant impairing effects on *D. magna* and *L. minor*, a reduced abundance of *L. variegatus* (by up to 46%) was observed compared to the medium control. Subsequent ozonation and additional filtration of the wastewater enhanced water quality, visible in an improved performance of *L. variegatus.* In general, direct evidence for the formation of toxic TPs due to the advanced wastewater treatments was not found, at least not in concentrations high enough to cause measurable effects in the investigated test systems. Additionally, no evidence for immunotoxic effects of the investigated wastewater effluents were observed. Yet, study-site- and species-specific effects hindered the definite interpretation of results. That underline the importance of a suitable test battery consisting of representatives of different taxonomic groups and trophic levels, to ensure a comprehensive evaluation of the complex matrix of wastewater and to avoid false-negative or false-positive results.

With aim to improve knowledge regarding immunotoxicity in invertebrates, the potential immunotoxic effects of the immunosuppressive pharmaceutical cyclosporine A (CsA) were investigated by applying the host-parasite model system *Daphnia magna – Pasteuria ramosa* in an adapted *host resistance assay.* Co-exposure to CsA and *Pasteuria* synergistically affected long-term survival of *D. magna*.

Additionally, the enhanced virulence of the pathogen upon chemical co-exposure was expressed in synergistically increased infection rates and an increased speed of *Pasteuria*-induced host sterilization. In conclusion, results provide evidence for a suppressed disease resistance in a chemically stressed invertebrate host, highlighting the importance of investigating the conjunction of environmental pollutants and pathogens in the environmental risk assessment of anthropogenic pollutants.

Zusammenfassung

Der zunehmende Konsum von Pharmazeutika, Körperpflegeprodukten und Industriechemikalien führt zu einer erhöhten Wasserverschmutzung und es besteht eine wachsende öffentliche und wissenschaftliche Besorgnis über das Vorkommen von anthropogenen Chemikalien in der aquatischen Umwelt. Oberflächengewässer aber auch Grundwasserreservoirs sind Lebensraum für zahlreiche aquatische Organismen und dienen gleichzeitig als Haupttrinkwasserquelle. Vor allem in Ballungsgebieten werden diese Wasserspeicher durch hauptsächlich organische Schadstoffe aus Kläranlageneinträgen belastet. Konventionelle Kläranlagen sind darauf ausgelegt, Nährstoffe, wie anorganische Stickstoffverbindungen und Phosphat zu reduzieren und relevante Schadstoffparameter, wie beispielsweise den chemischen und den biologischen Sauerstoffbedarf zu senken, sowie potentielle Krankheitserreger zu entfernen. Durch die Sorption an Klärschlamm wird zusätzlich die Abtrennung vieler unpolarer Stoffe realisiert, während die Mehrzahl der polaren Verbindungen in konventionellen Kläranlagen schlecht abbaubar ist. Herkömmliche Kläranlagen fungieren daher als ständige Punktquellen für die unbeabsichtigte Freisetzung von (Mikro-)Schadstoffen in die aquatische Umwelt. Anthropogene Mikroverunreinigungen sind mittlerweile in sehr geringen Konzentrationen in fast allen aquatischen Kompartimenten messbar und negative Auswirkungen, wie beispielsweise akute und chronische, sowie endokrine Effekte auf Wasserorganismen aber auch Antibiotikaresistenzen von Mikroorganismen konnten nachgewiesen werden. Bislang existieren keine regulatorischen Richtlinien für die Überwachung und den Eintrag der Vielzahl der anthropogenen Mikroschadstoffe und ihrer potentiellen Transformationsprodukte in aquatische Ökosysteme.

In Anbetracht der aktuellen Situation der Wasserverschmutzung und mit dem Ziel einer verbesserten Wasserqualität in Bezug auf die Umwelt und die menschliche Gesundheit, wurde in den letzten Jahren die Einführung fortschrittlicher Technologien zur Abwasserbehandlung, wie Ozonung als oxidatives und Aktivkohlefiltration als adsorptives Verfahren, intensiv diskutiert und untersucht.

Während Substanzen mithilfe der Aktivkohlefiltration durch Sorption entfernt werden, führt die Ozonung des Abwassers zu einem strukturellen Umbau der Substanzen, wobei nur die wenigsten dieser Verbindungen vollständig mineralisiert werden. Neben den Vorteilen der effizienten Entfernung einer Vielzahl von organischen Verbindungen sowie von Krankheitserregern aus dem Abwasser, wurde festgestellt, dass insbesondere die Abwasserbehandlung mit Ozon zur Bildung von weitestgehend unbekannten Transformationsprodukten mit oft unbekannter Toxizität führen kann. Neben anthropogenen Chemikalien, bedroht auch das Vorhandensein von Krankheitserregern, wie Viren, Bakterien oder Protozoen, die Qualität des verfügbaren Süßwassers und kann Risiken für Mensch und Umwelt darstellen. Neben einem direkten Infektionsrisiko, birgt die Interaktion von anthropogenen Mikroschadstoffen und Krankheitserregern Risiken, die gerade für aquatische Invertebraten bisher kaum untersucht wurden.

Bezugnehmend auf oben genannte Zusammenhänge untersuchte das vom Bundesministerium für Bildung und Forschung (BMBF) geförderte Forschungsprojekt *TransRisk* die "Charakterisierung, Kommunikation und Minimierung von Risiken durch neue Schadstoffe und Krankheitserreger im Wasserkreislauf". Im Rahmen dieses Forschungsprojektes konzentrierte sich die vorliegende Thesis auf die ökotoxikologische Untersuchung wasserbürtiger Schadstoffe, einschließlich ihrer potentiell toxischen Transformationsprodukte. Dazu wurden einerseits bereits identifizierte Transformationsprodukte ökotoxikologisch charakterisiert, andererseits wurden erweiterte Abwasserbehandlungen auf ihr Potential hin untersucht, zu einer veränderten Toxizität des Abwassers beizutragen. Ein weiterer Schwerpunkt lag auf der Untersuchung der kombinierten Wirkung von anthropogenen Schadstoffen und Krankheitserregern mit Auswirkungen insbesondere auf wirbellose Wasserlebewesen. Dazu wurde das Wirt-Pathogen-Modell *Daphnia magna* – *Pasteuria ramosa* verwendet, um in adaptierten *host resistance assays* die Auswirkungen potentiell immuntoxischer Verbindungen zu untersuchen.

Das Virostatikum Aciclovir (ACV) ist ein Beispiel für ein Humanpharmakon mit strukturell identifizierten Transformationsprodukten, die während der konventionellen Abwasserbehandlung und nach anschließender Ozonung entstehen. Vor Beginn dieser Thesis lagen keine ökotoxikologischen Daten bezüglich der Ausgangsverbindung und der beiden Transformationsprodukte vor. Die möglichen ökotoxikologischen Wirkungen der drei Verbindungen wurden daher mit *Raphidocelis subcapitata*, *Daphnia magna* und Embryonen von *Danio rerio* als Vertreter verschiedener trophischer Ebenen untersucht. Während die Ausgangsverbindung Aciclovir bei keinem der untersuchten Organismen zu einer akuten Toxizität führte, zeigten beide Transformationsprodukte eine erhöhte aquatische Toxizität. Während Carboxy-Aciclovir, das Bioabbauprodukt von Aciclovir, die Reproduktion von *D. magna* signifikant um 40% reduzierte (bei einer Konzentration von 102 mg/l), führte das Ozonungsprodukt COFA, zu einer signifikanten Wachstumshemmung der Grünalge *R. subcapitata* (EC_{10} = 14,1 mg/l). Die vorliegenden Ergebnisse verdeutlichen, dass erweiterte Abwasserbehandlung, und vor allem Ozonung, zur Bildung von polaren Transformationsprodukten führen kann, die unter Umständen eine höhere Toxizität gegenüber untersuchten Organismen aufweisen als die jeweilige Ausgangsverbindung. Die Ergebnisse unterstreichen die Notwendigkeit weiterer Forschung im Zusammenhang mit der Identifizierung und Charakterisierung von Transformationsprodukten, die bei erweiterten Abwasserbehandlungsprozessen entstehen können.

Um die mögliche Verringerung oder Verstärkung der toxischen Wirkung von neun unterschiedlich behandelten Abwässern zu untersuchen, wurden ausgewählte Tests mit *Daphnia magna*, *Lumbriculus variegatus* und *Lemna minor* in Durchfluss-Testsystemen auf einer Pilot-Kläranlage durchgeführt. Die verschiedenen Behandlungsprozesse beinhalteten Ozonung konventionell behandelten Abwassers mit anschließenden Filtrationsprozessen, sowie Membranbioreaktorbehandlung in Kombination mit Ozonung. Es wurde die Hypothese getestet, dass oxidative Prozesse, wie beispielsweise die Behandlung des Abwassers mit Ozon,

beeinträchtigende Effekte auf eingesetzte Organismen nach sich ziehen, und dass solche Effekte durch anschließende Filtrationstechniken reduziert werden können. Während die Exposition gegenüber konventionell gereinigtem Abwasser keine signifikanten Effekte auf *D. magna* und *L. minor* zur Folge hatte, wurde eine im Vergleich zur Negativkontrolle um bis zu 46% reduzierte Anzahl von *L. variegatus* beobachtet. Ozonung des konventionell geklärten Abwassers und zusätzliche Filtration führten zu einer verbesserten Wasserqualität, welche sich vor allem in der erhöhten Anzahl von *L. variegatus* zeigte. Erhöhte Nitrit- und Ammoniumwerte im Ablauf der Membranbioreaktoren (sowohl mit als auch ohne anschließende Ozonung) führten zu einer drastischen Reduktion der Abundanz von *L. variegatus*. Nach Exposition gegenüber GAK-gefiltertem Abwasser wurde ein signifikant reduziertes Wachstum von *L. minor* beobachtet, was eine Folge der Nährstoffentfernung durch Filtration sein kann.

Entgegen der Erwartungen wurde kein direkter Nachweis für die Bildung toxischer Transformationsprodukte nach erweiterter Abwasserbehandlung gefunden, zumindest nicht in Konzentrationen, die hoch genug waren, um messbare Effekte in den untersuchten Testsystemen zu verursachen. Studienort- und artspezifische Effekte, erschwerten jedoch die eindeutige Interpretation der Ergebnisse. Dies unterstreicht die Bedeutung einer geeigneten Testbatterie bestehend aus Vertretern verschiedener taxonomischer Gruppen und trophischer Ebenen, um eine umfassende Bewertung der komplexen Abwassermatrix zu gewährleisten und falsch-negative oder falsch-positive Ergebnisse zu vermeiden.

Mit dem Ziel, das Wissen über Immuntoxizität bei Wirbellosen zu verbessern, und die Eignung des gewählten Wirt-Pathogen-Modells *Daphnia magna – Pasteuria ramosa* zu überprüfen, wurde der Einfluss des humanen Immunsuppressivums Cyclosporin A (CsA) auf die Virulenz von *P. ramosa* in einem adaptierten *host resistance assay* untersucht. Es wurde vermutet, dass sich potentielle immunsuppressive Effekte der Modellsubstanz CsA in einer erhöhten Virulenz des Pathogens, und damit einhergehend in einer erhöhten Sterilisationsrate und verminderter Überlebensrate von *D. magna* äußert. Daphnien, die zu Testbeginn

weniger als 24 h alt waren, wurden für 21 Tage gegenüber CsA exponiert, wobei während der ersten 72 Stunden eine zusätzliche Exposition gegenüber dem Pathogen erfolgte. Die simultane Exposition gegenüber CsA und *Pasteuria* führte zu einem signifikant reduzierten Überleben von *D. magna*. Zusätzlich wurde eine erhöhte Virulenz des Pathogens in Anwesenheit von CsA beobachtet, die sich in synergistisch erhöhten Infektionsraten und einer erhöhten Geschwindigkeit der *Pasteuria*-induzierten Sterilisation von *D. magna* bemerkbar machte. Die Ergebnisse liefern eindeutige Hinweise, dass ein Arzneimittel, welches die humane Immunantwort unterdrücken soll, ebenfalls immuntoxische Wirkung auf wirbellose Organismen zeigen kann. Die Ergebnisse unterstreichen die Notwendigkeit, Immuntoxizität in der Umweltrisikobewertung zu berücksichtigen und geeignete standardisierte Methoden zu diesem Zweck zu entwickeln.

Zusätzlich wurde das gewählte Wirt-Parasit-System *D. magna* – *P. ramosa* zur Untersuchung des immuntoxischen Potentials von Abwässern nach konventioneller Behandlung, sowie nach anschließender Ozonung und nach Biofiltration eingesetzt. Dazu wurden juvenile Daphnien in einem Durchflusstestsystem für 21 Tage gegenüber den verschiedenen Abwässern exponiert. Die Exposition in den ersten 72 Stunden erfolgte statisch und mit zusätzlicher Exposition gegenüber dem Pathogen *P. ramosa*. Keines der untersuchten Abwässer lieferte Hinweise auf ein immuntoxisches Potential. Es wurde vielmehr eine im Vergleich zur *Pasteuria*-Kontrolle reduzierte Infektionsrate der Daphnien nach kombinierter Exposition gegenüber Abwasser und *P. ramosa* beobachtet.

Die Ergebnisse der vorliegenden Thesis unterstreichen die Wichtigkeit der Auswahl relevanter Testsysteme und zu untersuchender Endpunkte, um einseitige und möglicherweise falsch-positive oder falsch-negative Ergebnisse zu vermeiden und unter anderem auch immuntoxische Potentiale zu erkennen.

1 General introduction

Water is one of the greatest goods for human kind and the environment. It covers two thirds of the earth's surface, composes essential parts of all living organisms and is fundamental for life on earth. According to the United Nations, clean drinking water and sanitation are human rights and should therefore be made accessible to the entire world population. However, there is concern, that by 2040 only 70% of the total global water demand will originate from natural sources and already today more than 2 billion people have no access to safe drinking water and sanitation (UN-Water, 2018). The worldwide expanding population density and the associated increasing consumption of pharmaceuticals and personal care products (PPCPs) and industrial chemicals are resulting in an increase in water pollution, with potential risks for human health and still unknown ecotoxicological long-term impacts on aquatic organisms.

More than 143 million organic and inorganic chemical substances are currently identified and listed in the American Chemical Society's Chemical Abstracts Service (CAS) Registry and this enormous number continues to grow – daily, approximately 15,000 chemicals are added to the list (www.cas.org). Since chemicals are used ubiquitous in everyday life (in industrial processes and products, as pesticides and biocides, as flame retardants, in human and veterinary pharmaceuticals and in personal care and cleaning products to only name a few) a huge amount of these anthropogenic pollutants is ending up in surface and even drinking waters. For the majority of these substances and their possible emerging transformation products (TPs), there is no or only insufficient information available regarding potential hazards to humans and the environment (Daughton & Ternes, 1999; Eggen et al., 2014).

Besides anthropogenic chemicals, the presence of waterborne pathogens, such as viruses, bacteria or protozoans is threatening the quality of available freshwater and may pose risks for humans and the environment (Ross, 2010; Rizzo et al., 2013). In addition to the direct risk of infection, the interaction of anthropogenic micropollutants and pathogens may pose risks that have barely been studied, especially for invertebrate aquatic organisms.

1.1 Scope of the present thesis

One of the key aspects of the present thesis was the investigation of aquatic toxicity of anthropogenic emerging contaminants, including their potential TPs, which may be present in ozonated wastewater. The second focus was laid on the investigation of possible joint effects of anthropogenic contaminants and pathogens with effects especially on aquatic invertebrate organisms. Research was financially supported by the Federal Ministry of Education and Research (German: Bundesministerium für Bildung und Forschung - BMBF) within the joint research project TransRisk (FKZ: 02WRS1275F, duration 11/2011 – 04/2015) which aimed at the "characterization, communication and minimization of risks originating from emerging contaminants and pathogens in the water cycle". The concerns regarding possible hazards of anthropogenic contaminants and their TPs as well as the conjunction of xenobiotics and pathogens with regard to aquatic organisms are addressed in the following chapters.

1.2 Anthropogenic micropollutants

Todate anthropogenic micropollutants, i.e. substances that are detectable in very low concentrations, such as the ng to µg L^{-1} range, are measurable in almost every compartment in the aquatic environment (Lapworth et al., 2012; Margot et al, 2013).
There are a variety of pathways for micropollutants to enter surface waters, most of them related to human activities, such as industrial production, agricultural activities, use of PPCPs or cleaning products. Most of the detectable anthropogenic micropollutants are very persistent and their removal during wastewater treatment, mainly by sorption to suspended solids, may be limited by their polarity (Ternes et al., 2004; Behera et al., 2011).
For these reasons, there is concern that municipal wastewater treatment plants (WWTPs), which were in fact originally designed for the removal of nutrients and non-polar chemical compounds, are not or only partially able to remove the variety of (polar) organic compounds from today's wastewater (Janssens et al., 1997; Bolong et

al., 2009; Gros et al., 2010) and thus serve as significant and continuous sources for the release of micropollutants into surface and even ground waters and though into the aquatic environment (e.g. Choubert et al., 2011; Deblonde et al., 2011; Lapworth et al., 2012; Prasse et al., 2015). A variety of anthropogenic micropollutants has been detected in water cycles globally (Schwarzenbach et al., 2006; Kasprzyk-Hordern et al., 2009; Regnery & Püttmann, 2010; Spongberg et al., 2011) and WWTP-discharges have already been linked to adverse environmental effects on aquatic species including acute and chronic toxicity, endocrine disrupting effects as well as antibiotic resistance of microorganisms (Fent et al., 2006; Pruden et al., 2006; Ashauer, 2016). To date, no limiting regulatory guidelines exist regarding discharge and monitoring of the variety of micropollutants and their potential by-products (Bolong et al., 2009). The European Commision has been adressing this issue by adopting the Water Framework Directive (WFD) (2000/60/EC), and by listing 33 priority substances for monitoring of the chemical status of surface and ground waters. In 2013 the Environmental Quality Standards Directive (2008/105/EC) added 12 additional priority substances. Yet, most likely these priority substances do not necessarily represent the most problematic substances present in the aquatic environment.

1.3 Advanced wastewater treatment

Initially conventional WWTPs were designed for the removal of nutrients, such as nitrogen, phosphorus and total organic carbon (TOC), for the decrease of relevant pollution parameters (such as the chemical oxygen demand (COD), the biological oxygen demand (BOD) or the load of total suspended solids (TSS)) and for elimination of pathogens and coliforms. By sorption to sewage sludge the removal of many non-polar substances is realized additionally, whereas the majority of the more recalcitrant, polar compounds is poorly degradable within conventional WWTPs (Ternes & Joss, 2004). Conventional WWTPs usually consist of a preliminary treatment (for the removal of coarse solids and other large materials), a subsequent mechanical pre-treatment (for the removal of settleable organic and inorganic solids by sedimentation and the removal of floatable materials by skimming), followed by secondary biological treatment (e.g. activated sludge treatment) with nitrification and denitrification steps.

Residual organics and suspended solids are usually removed in a final clarification step.

With regard to the current situation of water pollution and related to the requirements of the European WFD, which aims at achieving a good ecological status of inland and coastal waters as well as groundwaters until 2027 (originally target dates were 2015 (1st agreement) and 2021 (2nd agreement)), the upgrade of conventional WWTPs with tertiary treatment processes, such as different filtration techniques (e.g. activated carbon), advanced oxidation processes (AOPs) such as ozonation, or membrane bioreactor (MBR) treatment processes became more important in recent years (e.g. Eggen et al., 2014; Luo et al., 2014). Large-scale trials already provided evidence for the successful removal of a broad range of target micropollutants (Hollender et al., 2009; Margot et al., 2013).

Since tertiary treatment processes are usually associated with high energy consumption and expensive treatment costs, the decision to implement such advanced treatment processes is always related to national environmental and public health policies and are often bound to long term decision processes. To date, several countries focus on the implementation of advanced wastewater treatment processes, yet Switzerland still pioneers with their policy to upgrade ~100 of their WWTPs within the next ~20 years (Eggen et al., 2014).

Due to the heterogeneity of the vast group of micropollutants, it is challenging to find a universal treatment solution. However, considering removal of hydrophilic organic compounds and energetic as well as cost-balances, activated carbon treatment and oxidation processes currently seem to be the most promising tertiary treatments following conventional activated sludge treatment (Ternes & Joss, 2007; Joss et al. 2008). Within the research project *TransRisk* ozonation of wastewater in combination with different (subsequent) processes was investigated for the treatment of municipal wastewater with aim to improve removal of anthropogenic micropollutants and pathogens. The treatment processes were installed in pilot-plant scale on a pilot treatment plant in Southern Hesse, receiving real wastewater from a WWTP with municipal and industrial origin (about 70% and 30% respectively). In addition to conventional biological treatment, ozonation with subsequent filtration processes (activated carbon and biofilter treatment) (Fig. 1) as well as MBR treatment in

combination with ozonation were investigated (Fig. 2). The resulting nine different effluents were ecotoxicologically evaluated in the present thesis using an *in vivo* test battery consisting of aquatic representatives of different taxonomic groups. Respective results are presented in the Annex I.II.

The combined treatment processes are in detail described by Knopp et al. (2016) and shortly addressed in the following subchapters.

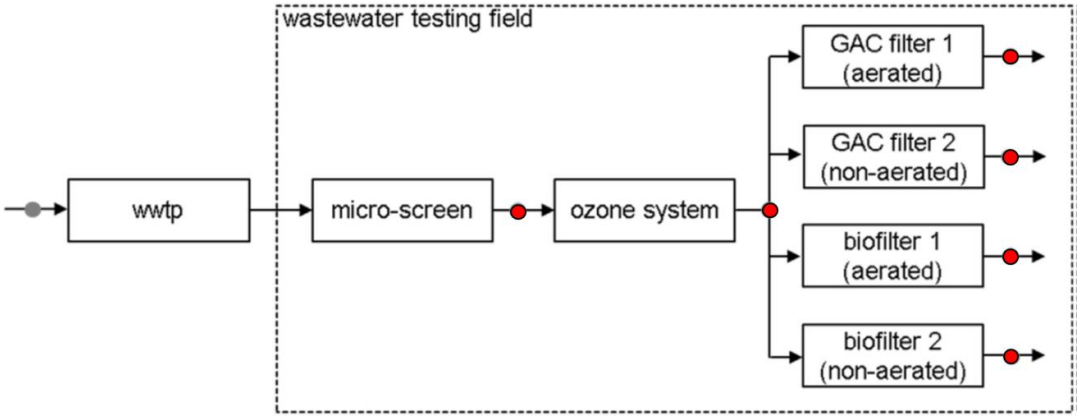

Figure 1: Schematic overview of the investigated advanced treatment processes related to the 1st ozone system with subsequent filtration techniques. GAC – granulated activated carbon. Effluents investigated in the *in vivo* test systems are indicated by red dots. (Reprint from Knopp et al., 2016)

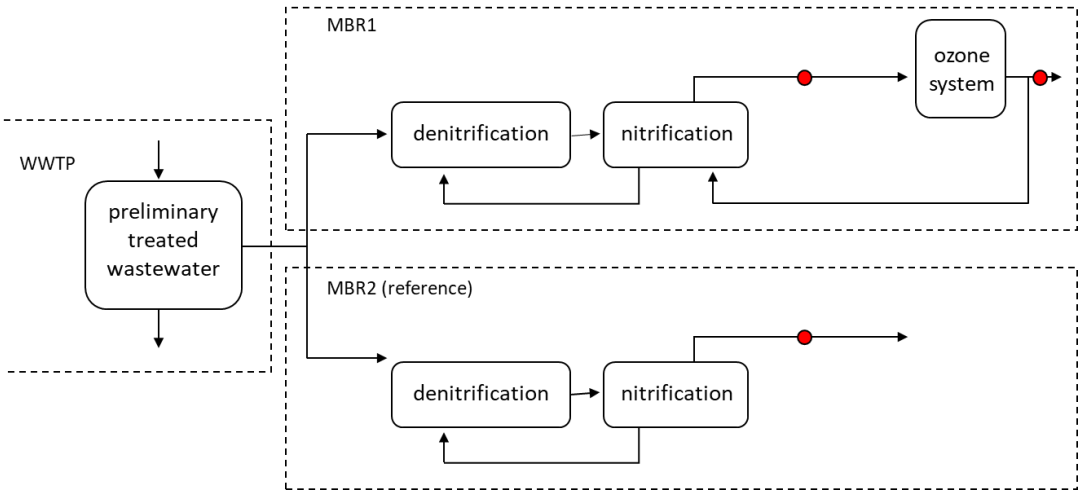

Figure 2: Schematic overview of the investigated advanced treatment processes related to the MBR treatments and the 2nd ozone system. Effluents investigated in the *in vivo* test systems are indicated by red dots.

Ozonation

Ozone (O_3) is a powerful oxidizer with high elimination rates of microorganisms and organic contaminants for which adequate elimination by adsorption or conventional biological treatment is not provided (Janex et al., 2000; Snyder et al., 2006). Ozone is unstable in water and degrades into hydroxyl radicals (HO·), which are unspecific and powerful oxidizing radicals, that can react with a variety of dissolved compounds in the water matrix. Ozone itself reacts more selectively for electron-rich chemicals, e.g. compounds with double bonds (Acero & von Gunten, 2001; von Gunten, 2003a; Snyder et al., 2006).

Activated carbon filtration

Adsorption by activated carbon, both in granulated (GAC) and in powdered (PAC) form, is an efficient method to remove a broad range of dissolved organic compounds with non-polar characteristics and matching pore size and shape properties (Ghosh et al., 1999; Matsui et al., 2002a, 2002b; Rossner et al., 2009;). Activated carbon is applicable in secondary as well as in tertiary treatment processes and is commonly related to the elimination of taste-, odor- and color-promoting compounds (Knappe et al., 1998; Luo et al., 2014). The main advantage of activated carbon treatment is the high elimination rate of micropollutants due to accumulation onto the solid phase, and thus reducing or even eliminating the risk of formation of toxic by-products. However, some polar components, such as the anticonvulsant drug gabapentin, are not completely eliminated by activated carbon treatment (Reungoat et al., 2010), pointing to the efficiency limitation of this treatment method.

Biofilter processes

In biofiltration processes, microorganisms are used for the purification of wastewater. Within the biofilm, the microorganisms live in close proximity and form symbiotic biocenoses, which are able to degrade even the more recalcitrant substances in the wastewater. A constant inflow of nutrients and organic matters is essential for the microorganisms and their effective functionality (Chaudhary et al., 2003). In addition to the biodegradation of organic compounds and nutrients, a retention of suspended solids is optimally realized.

Membrane bioreactor

A combination of conventional activated sludge treatment and membrane filtration is given in MBR processes, which is beneficial with regard to higher solid contents in the reaction chamber, higher rates of microbial separation due to higher solids retention time and high effluent quality (Spring et al., 2007; Luo et al., 2014). MBRs were shown to be efficient in the removal of compounds, such as pharmaceuticals, that were not eliminated in conventional activated sludge treatment (Radjenović et al., 2007, 2009).

1.4 Transformation products of anthropogenic micropollutants

The main advantage of advanced wastewater treatment methods, such as activated carbon filtration or treatment with ozone, is the efficient removal of a variety of recalcitrant, organic compounds as well as pathogens and coliforms to a much greater extent than observed in conventional biological treatment (Huber et al., 2005; Hollender et al., 2009; Reungoat et al., 2010, 2012; Margot et al., 2013).

Yet, whereas complete mineralization of compounds during wastewater treatment is scarce, as for example demonstrated for aspirin (Richardson & Bowron, 1985), many compounds are not completely mineralized, but rather transformed into a variety of largely unknown TPs with often unknown toxicity and unknown threats for humans and the environment (Schulz et al., 2008; Oulton et al., 2010; Zhang & Li, 2011; Hübner et al. 2014). Often there is more than one by-product formed, as for example demonstrated for the antiepileptic drug carbamazepine, or the beta blocker propranolol (McDowell et al., 2005; Benner & Ternes, 2009a).

While ozonation, as an effective tool for the removal of pathogens and anthropogenic contaminants, is one of the most promising advanced treatment methods, it is simultaneously known to be associated with the formation of ozonation by-products (Richardson et al., 2007). A well-known example is the microbial degradation of the fungicide tolylfluanide to the non-toxic decomposition product N,N-dimethylsulfamide (DMS), which is transformed into the highly carcinogenic N-nitrosodimethylamine (NDMA) during ozonation (Schmidt & Brauch, 2008). Another example is the antiviral drug acyclovir (ACV), which is used for the treatment of herpes infections and has been detected in German WWTP influents in concentrations up to 2 µg/l (Prasse et al., 2011).

Aerobic degradation of ACV in the WWTP, due to the oxidation of the terminal hydroxyl group leads to the formation of the microbiologically very stable TP carboxy-acyclovir (C-ACV). C-ACV was detected in German rivers in concentrations up to 3.2 µg/l. C-ACV is almost completely eliminated after treatment with ozone. However, instead of degradation of the compound, the formation of another stable TP (N-(4-carbamoyl-2-imino-5-oxoimidazolidine)formamido-N-methoxy-acetic acid, in the following called COFA) is reported (Prasse et al., 2011, 2012). In general TPs often have an only slightly altered chemical structure but can be very stable and, in some cases, have a similar or even higher (eco-) toxicological active potential than their parent compound (Boxall et al., 2004).

In the present thesis, the potential ecotoxicological effects of a pharmaceutical and its TPs on different representatives of aquatic organisms were investigated using the example of ACV and its TPs (Annex I.I).

1.5 Regulatory background

Regarding chemical safety assessment, the European regulation concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH, regulation No 900/2014) controlls production, import and use of chemicals. The quality of surface waters and groundwater is addressed among others in the European Framework Directive (WFD, 2000/60/EC), the Federal Water Act (Wasserhaushaltsgesetz, WHG) or the German Surface Waters Ordinance (Oberflächengewässerverordnung, OgewV).

REACH was initiated in 2007 (and subsequetly officially terminated on May 31, 2018) and aimed to ensure a high level of protection of human health and for the environment. The chemical safety assessment starts with a hazard assessment for humans and the environment, based on the physical and chemical properties of the substance. Among others, it investigates if the substance has *persistent, bioaccumulative and toxic* (PBT) or *very persistent and very bioaccumulative* (vPvB) properties. Subsequently, threshold concentrations for the environment and human health are derived. The Predicted No-Effect Concentration (PNEC) indicates the concentration of the substance in the environment at which no harmful effects are

expected. If a substance is classified as hazardous, or if it has PBT or vPvB properties, the registrant has to additionally estimate the Predicted Environmental Concentration (PEC) in an exposure assessment. Chemicals that are poorly soluble in water and/or easily soluble in fat, often exhibit no acute toxicity but may cause effects in long-term exposure scenarios such as increased mortality and reduced growth or reproduction. Therefore, data on long-term toxicity for invertebrates (Daphnia, REACH Annex IX, 9.1.5) and fish (REACH Annex IX, 9.1.6) are required for substances with chemical properties of concern (e.g. low water solubility) and for substances that exceed a production or import quantity of 100 t/year.

The **WFD** (2000/60/EC) aims to regulate the chemical, ecological and hygienic quality of all water bodies including surface and groundwater on the European level. A list of environmental quality standards for, to date, 45 priority substance was applied in the Environmental Quality Standards Directive (2008/105/EC), that mainly includes industrial chemicals as well as pestizides and biocides and defines threshold concentrations, which are considered to be unharmful for aquatic organisms, and that should not be exceeded in surface waters.

The most important federal law on German level is the WHG, that aims to achieve good quality status for all water bodies until 2027, with regard to minimized pollutant levels and good ecological status for native aquatic species (animals and plants). The central objectives and management rules are implemented according to the WFD, and are executed due to "Subsidiary Directives" such as the OGewV. The **OGewV** controls emissions and discharges of priority substances and selected pollutants and implements EU legal provisions onto German law, such as the previously mentioned directives 2000/60/EC and 2008/105/EC.

To date most emerging micropollutants as well as their potential TPs are currently not covered by existing environmental regulatory edicts. A few pharmaceuticals, such as the analgesic drug diclofenac, the hormones 17alpha-ethinylestradiol and 17beta-estradiol, as well as three macrolide antibiotics have been listed on a watch-list of emerging aquatic pollutions by the European Commission, for systematic monitoring across Europe. This aims at the identification of the environmental impact, related to

these substances and to highlight the need to include micropollutants, such as pharmaceuticals in the list of priority substances according to the WFD.

The regulatory assessment of TPs is currently only covered in the case of pesticide metabolits in the Pesticide Directive (1107/2009/EC), all other regulation directives do not involve TPs in their regulatory assessment. Besides TPs, effects of contaminant mixtures are also not covered in the regular regulatory edicts and toxicity assessment of chemicals still focus mainly on the investigation of individual substances (Beyer et al., 2014).

1.6 Immunotoxicity of anthropogenic micropollutants

Since pathogens are ubiquitous in almost every habitat, an intact immune system is inevitable for the protection of organisms and populations (Schmid-Hempel, 2011; Blaustein et al., 2012). In recent decades concern has grown about anthropogenic (micro-)pollutants that exhibit no or only marginal toxicity but may act as immunotoxicants and thereby disturb the immune function of both humans and wildlife.

For various wildlife species, contamination of their habitats was discussed to be linked to an increasing susceptibility to infectious diseases. Respective contaminants can be effective even in very low concentrations and yet drastically minimize populations (Mason et al., 2013). That was for example seen in 1988 and 2002, when a total of approximately 53,000 wild seals (*Phoca vitulina*) died from *phocine distemper virus* (Härkönen et al., 2006). For the mass mortality of 1988, among others, the accumulation of polychlorinated biphenyls (PCBs) and dichlorodiphenyldichloroethylene (DDE) in the examined tissue of infected seals was held responsible for the increased susceptibility to the pathogen. In 2002 PCBs and DDE could hardly be detected in seal tissue, however “new” substances, such as organotin compounds or brominated flame retardants, were assumed to have caused observed effects in the affected seals (Härkönen et al., 2006).

Substances that may exhibit immunotoxic properties can be associated to a broad range of chemical classes (Galloway and Depledge, 2001). Among others, immunosuppressive effects have been demonstrated for heavy metals (Pipe & Coles,

1995; Parry & Pipe, 2004; Sorvari et al., 2007), pesticides (Gagnaire et al., 2007; Coors et al., 2008; Cerbin et al., 2010; Coors & De Meester, 2011; De Coninck et al. 2013), industrial chemicals (Coles et al., 1994) and complex mixtures such as wastewater effluents (Gagné et al., 2007, 2008). Pharmaceuticals with intentionally immunosuppressant efficiencies, that enter the aquatic environment mainly due to improper elimination in the WWTPs (Kümmerer, 2009) may also pose serious threats to affected organisms (Gagné et al., 2006). These drugs are mainly prescribed to treat autoimmune diseases and to prevent allograft rejections. The consumption of immunomodulatory substances in Germany increased between 2002 and 2009 by 91% (from 6,799 kg to 13,005 kg) (Bergmann et al., 2011). Assuming an increased release of these immunomodulatory pharmaceuticals into all sorts of water bodies and thus into the aquatic environment, potential immunotoxic effects of pharmaceuticals in particular and anthropogenic micropollutants in general should be considered with regard to aquatic organisms.

In the present thesis, the potential immunotoxic effects of the immunosuppressant drug cyclosporine A (CsA) were investigated on *D. magna*. CsA affects the innate as well as the adaptive immune system and is applied after transplants in humans to avoid organ rejections. CsA is a calcineurin-inhibitor and has also antibiotic as well as antifungal properties (Kümmerer, 2004). It was previously demonstrated to affect immune functions, such as the inhibition of phagocitic, cytotoxic and antibacterial peptides activity in some invertebrate species (Vilcinskas et al., 1999; Fiolka, 2008, 2012).

Immunotoxicity in invertebrates

The attention regarding immunotoxicity has focused on vertebrates, especially mammals so far, while, despite their ecological importance, little information is available regarding immunotoxicity in invertebrates. Although invertebrates represent the vast majority (approximately 95%) of all species worldwide and play a vital functional role in the ecosystem, immunotoxicity is still a regulatory endpoint only with regard to toxicological but not to environmental risk assessments (ERA). Due to their importance in the ecosystem, invertebrates in particular require further attention to the toxic effects of environmental pollutants and additional (biotic) stressors. For

this reason, it is necessary to examine causal relationships between exposure to pollutants and the impairment of the immune system by means of experimental and multifactorial investigation approaches (Galloway and Depledge, 2001).

Unlike vertebrates, invertebrates possess, as far as it is known, no adaptive immune system but only a nonspecific, so-called innate immune system similar to that of vertebrate organisms (Söderhäll & Cerenius, 1998). However, evidence of memory and specific immune functions, which are characteristics of the vertebrate immune system, were found even in some of the most basic invertebrate phyla such as Coelenterata and Cnidaria (Hildemann et al., 1977, 1979; Bigger et al. 1982). The main function of the innate immune system is the recognition of different cell types and the distinction between self and non-self agents to defend the organism against pathogenetic microorganisms.

Using various measurements of general and specialized immune parameters (including measurements of cell viability, phagocytosis activity, number and activity of hematocytes, cytotoxicity assays, cytokines, macrophages, natural killer cells) possible immunosuppressive substances may be identified (Luebke et al., 2007). Yet so-called *host resistance assays*, where the resistance or susceptibility to pathogens is tested under the simultaneous influence of a chemical contaminant, serve as "gold-standard" for the investigation of immunotoxic effects (Descotes, 2006). A decreased clearance of an infectious pathogen or otherwise, an increased virulence serve as distinct evidences for a demonstrated immunotoxicity of an investigated substance (Burleson & Burleson, 2008).

Due to the inherent redundancy of the immune system, an experimental approach with *in vivo* experiments is indispensable for the confirmation of causal relationships between susceptibility to infection (and disease progression) and exposure to pollutants. Thus, *host resistance assays* seem to be a promising tool for the investigation of immunotoxicity in invertebrates. To date a considerable number of papers has been published in which an increased susceptibility of invertebrates to pathogens has been correlated with the influence of environmental pollutants in *in vivo* test systems (e.g. Chou et al., 1998; Shirakashi & El Matbouli, 2010; Coors & De Meester, 2008, 2011; De Coninck et al., 2013). However, no standardized test systems for the detection of immunotoxic effects in invertebrate organisms have been developed so far.

One host-parasite combination, that has been extensivley studied in recent years, with regard to ecological, epidemiological and genetic interactions, is the host-parasite model of *Daphnia magna* and *Pasteuria ramosa* (Ebert et al., 1998, 2016; Regoes et al., 2003; Luijckx et al., 2011). The planktonic crustacean *D. magna* Straus is frequently used as model organism in standard tests of aquatic ecotoxicology. It reproduces via cyclic parthogenesis and is conctantly filter-feeding. The gram-positive, endospore-forming bacterium *P. ramosa* is one of its obligate parasites (Ebert et al., 1996, Ebert, 2005). Host infection takes place strictly horizontally by ingestion of endospores during filter-feeding and *P. ramosa* infects *Daphnia* hemolymph and muscles (Luijckx et al., 2011). The infection is microscopically and at an advanced stage also macroscopically visible (Fig. 3).

Figure 3: Image of *Pasteuria*-infected (left) and uninfected *Daphnia magna* (right). Age of daphnids: 21 days. *P. ramosa* spores appears as a dark mass that fills the entire body cavity of the host. Additionally, the brood chamber of the infected *Daphnia* is empty, indicating sterilization by *P. ramosa*, whereas the healthy female carries some embryos in the brood chamber. Source: L. Schlüter-Vorberg, ECT.

Joint effects of *P. ramosa* and potential immunotoxic chemical substances on *D. magna* were demonstrated in several studies applying modified *host resistance assays* (Coors et al., 2008; Coors & De Meester, 2008, 2011; Buser et al., 2012; De Coninck et al., 2013). The host-parasite system *D. magna* – *P. ramosa* appears to be a promising model for the investigation of immunotoxicty in invertebrates due to high specific as well as distinct infection responses (Carius et al. 2001; Luijckx et al., 2011).

In the present thesis, joint effects of *P. ramosa* and the model substance CsA were investigated on *D. magna* (Annex I.III). Additionally, potential immunotoxic effects of wastewater samples from different advanced treatment methods, such as ozonation and biofiltration, were investigated using the model system *D. magna* – *P. ramosa* (Annex I.II).

1.7 Integration of the present work into the current state of research

The presence of anthropogenic micropollutants and pathogens in the water cycle is of great concern for those responsible in the water industry and to consumers of drinking water and also poses potential risks to aquatic ecosystems (Jekel et al., 2013; Richardson & Ternes, 2014; Stamm et al., 2015). In recent years, much research was done to improve knowledge and to develop technical solutions for the removal of the broad range of anthropogenic micropollutants during wastewater treatment (e.g. Rosal et al., 2010; Jelic et al., 2011; Ratola et al., 2012; Loos et al., 2013). Simultaneously great effort was made regarding the investigation of emergence of TPs formed during conventional biological and advanced wastewater treatment methods and their occurrence and effects in the aquatic environment. These include experimental studies and sampling campaigns on effluents of conventional and advanced treatment plants to identify and characterize various pharmaceutical compounds (Jelic et al., 2011, Reungoat et al., 2012), kinetic degradation studies of selected pharmaceuticals on the influence of different disinfection methods (Salgado et al., 2013) as well as literature studies (Farré et al., 2008; Fatta-Kassinos et al., 2011, Escher & Fenner, 2011).
While there is also evidence on TPs formed during biological wastewater treatment (Zwiener et al., 2002; Quintana et al., 2005; Prasse et al., 2011), the main research focus (and main concern) relates to the formation of TPs as a consequence of AOPs (e.g. Huber et al., 2003; Petala et al., 2008; Oller et al., 2011; Prasse et al., 2012). According to previous studies, TPs of pesticides and biocides are in most cases less toxic than their parent compound, but in some cases may also exhibit higher aquatic toxicity (Boxall et al., 2004; Rosal et al., 2009; Stalter et al., 2010a, b). Prior to the start of the present thesis very little information was available for TPs of typical wastewater-borne pollutants such as PPCPs.

Related to the topic of immunotoxicity of anthropogenic micropollutants, several publications have been published in recent years, which investigated immunotoxic effects of pollutants on invertebrates, thus highlighting the relevance of this issue. In all these studies, the interaction of investigated pollutants and pathogens could be demonstrated, resulting in negative effects on respective host organisms. So far, there have been studies demonstrating immunosuppressive effects of environmental pollutants on invertebrates at the cellular level (e.g. Fang et al., 2013; Brandt et al., 2016), by focusing on population relevant endpoints, such as host survival or infection status (e.g. Buser et al., 2012; Minguez et al., 2012; Wu et al., 2012; Aufauvre et al., 2012; Pettis et al, 2012; De Coninck et al., 2013) as well as literature reviews (Renault, 2015). A variety of host organisms, pathogens and substances have been investigated, however, there are still large knowledge gaps in this area and there are still no standardized ecotoxicological methods available for the ultimate detection of an immunotoxic effect of pollutants on invertebrates.

Related to the issues mentioned above and arising from the current state of knowledge the joint research project TransRisk (FKZ: 02WRS1275F), funded by the BMBF, was initiated in 2011 to combine (eco)toxicological, chemical and technical approaches to develop strategies for the characterization and minimization of risks associated with effects of organic micropollutants and pathogens present in urban water cycles.

The experiments conducted in the present thesis related to the ecotoxicological investigation of the antiviral drug ACV and two of its known TPs and their effects on three different aquatic organisms were based on previous studies published by Prasse et al. (2011, 2012). Investigations presented by Stalter et al. (2010b) and Magdeburg et al. (2012) and the results obtained in these experiments served as the basis for the on-site tests carried out in the course of the present thesis to ecotoxicologically characterize differently treated wastewater streams and for the selection of the *in vivo* test battery. The experimental investigation of immunotoxicity of wastewater and cyclosporine A on *D. magna* in the presence of *P. ramosa* were based on studies by Coors et al. (2008, 2011).

1.8 Objectives and hypotheses of the present thesis

The present thesis aimed at the ecotoxicological investigation of emerging waterborne contaminants, their potential TPs formed during advanced wastewater treatment processes and their effects on different aquatic organisms. Additionally, joint effects of anthropogenic pollutants and *P. ramosa* as a selected *Daphnia* pathogen were investigated on *D. magna* as an invertebrate host model. The different investigated hypotheses are summarized here:

1. *Ecotoxicological investigation of acyclovir and two of its known transformation products*

   The antiviral drug ACV, is an example of a pharmaceutical with structurally identified TPs emerging during conventional wastewater treatment and after subsequent ozonation. Yet, prior to this study, no ecotoxicological data were existing regarding the parent compound and the two TPs. The potential ecotoxicological effects of ACV and its by-products C-ACV and COFA were therefore investigated with *Raphidocelis subcapitata, Daphnia magna* and embryos of *Danio rerio* as representatives of different trophic levels.

   Based on previous findings regarding toxification of by-products after oxidation processes, it was assumed that at least the oxidation product may exhibit a greater toxicity compared to the parent compound. Findings related to the ecotoxicological investigation of ACV and its TPs are presented and discussed in the related publication provided in Annex I.I.

2. *Investigation of differently treated wastewater streams on-site on a pilot plant in southern Hesse*

   By using selected *in vivo* bioassays with *Daphnia magna*, *Lumbriculus variegatus* and *Lemna minor*, the potential reduction or enhancement of toxic effects of nine differently treated wastewater effluents were examined on-site on a pilot WWTP within the present thesis.

   The hypothesis was tested, that oxidation treatment methods such as ozonation may lead to a toxification resulting in impairing effects on affected organisms, and that such effects may be reduced due to subsequent filtration techniques.

   Results related to above mentioned topic are presented and discussed in the related publication provided in Annex I.II.

3. *Investigation of the potential immunotoxicity of cyclosporine A and of differently treated wastewater effluents on Daphnia magna in the presence of Pasteuria ramosa*

   The potential immunotoxic effects of the immunosuppressive pharmaceutical cyclosporine A on an invertebrate host organism was investigated using the host-parasite model system *D. magna – P. ramosa* in an adapted *host resistance* assay. Aim was to improve knowledge regarding immunotoxicity in invertebrates, and to further explore the potential of the chosen system as invertebrate *host resistance assay*. Additionally, the chosen host-parasite system was applied for the investigation of the potential immunotoxicity of differently treated wastewater effluents.

   The hypothesis was tested that immunosuppressive effects of the model substance CsA and the tested wastewater effluents will result in an increased virulence of the model pathogen *P. ramosa*, leading to an enhanced sterilization rate and reduced survival of *D. magna*. Results regarding the investigation of immunotoxicity of the wastewater samples are provided in Annex I.II, results regarding immunotoxic effects of CsA on *D. magna* are provided in the manuscript in Annex I.III.

2 General discussion

2.1 Main findings

Annex I.I, Schlüter-Vorberg et al., 2015

The ecotoxicological investigation of acyclovir (ACV) and two of its known transformation products (C-ACV and COFA) was conducted to detect potential impairing effects of pharmaceutical TPs with known presence in the environment on three aquatic organisms (*Daphnia magna*, *Raphidocelis subcapitata*, embryos of *Danio rerio*). The main findings are:

- The parent compound ACV did not cause acute toxicity on the three aquatic organisms up to a tested concentration of 100 mg/l
- C-ACV, the biodegradation product of ACV, significantly reduced reproduction and intrinsic growth rate of *D. magna* by 40% and 22% respectively at 102 mg/l C-ACV
- The ozonation product COFA significantly inhibited growth of green algae *R. subcapitata* (EC_{10} = 14.1 mg/l) as shown in Fig. 4.

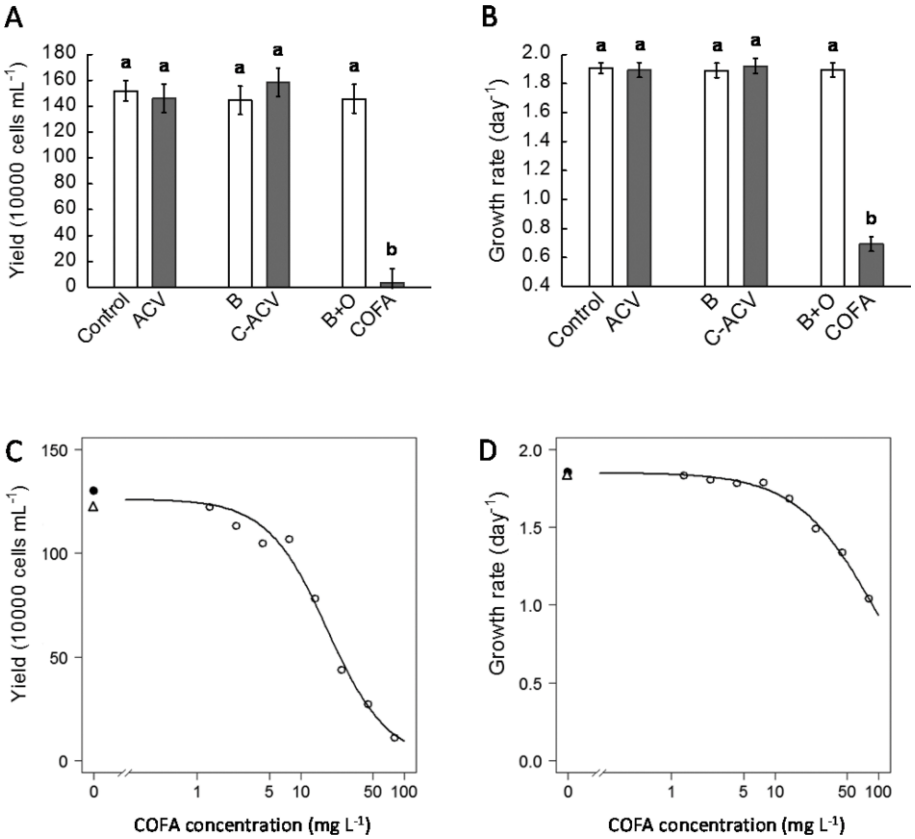

Figure 4: (A) Yield and (B) growth rate of *Raphidocelis subcapitata* after exposure for 72 h to ACV, its transformation products in the C-ACV and COFA treatments, the respective process control treatments B and B+O, and the control medium. Shown are mean responses with their 95% confidence intervals. Identical letters denote treatments that did not significantly differ from each other (Tukey HSD test; $\alpha = 0.05$). Concentration−response curves for (C) yield and (D) growth rate of *R. subcapitata* after exposure for 72 h to dilutions of the COFA treatment (based on measured COFA concentrations). Shown are means per treatment fitted by a three-parameter log−logistic model: (△) laboratory control, (●) process control treatment (B+O), and (○) COFA treatments.

- Neither the parent compound ACV nor the TPs C-ACV or COFA caused lethal or sublethal effects on embryos of *D. rerio*, indicating no acute fish toxicity of the tested compounds up to a concentration of 100 mg/l

Overall, ozonation as a method for directed elimination of anthropogenic micropollutants was shown to pose risks of formation of polar TPs with measurable toxicity towards aquatic organisms.

Annex I.II, Schlüter-Vorberg et al., 2017

The differently treated wastewater streams were ecotoxicological investigated on-site on a pilot plant. The aim was to compare different wastewater treatment processes regarding potential reduction or enhancement of toxic effects due to the elimination of micropollutants or the emergence of possible toxic TPs respectively. The potential effects of the differently treated wastewater effluents were investigated using three aquatic organisms (*L. variegatus, D. magna and L. minor*). Additionally, the immunotoxic potential of selected wastewater effluents was investigated, using the host-parasite model *D. magna – P. ramosa*. The main findings are summarized in the following:

- The conventional biological treated wastewater obviously still contained active compounds in environmentally effective concentrations, resulting in a reduced abundance of *L. variegatus* (by up to 46%) compared to the medium control.
- Ozonation and subsequent filtration of wastewater improved performance of *L. variegatus*
- Elevated levels of nitrite and ammonium in the effluent of the MBRs (both with and without subsequent ozonation) caused drastic reduction of *L. variegatus* abundance during the first exposure period
- Exposure to the conventionally treated wastewater did not result in significant impairing effects on *D. magna* and *L. minor*
- GAK filtered wastewater negatively affected growth of *L. minor*, which may be a consequence of nutrient removal

Table 1: Summary of the results of the on-site *in vivo* tests regarding the investigation of the differently treated wastewaters of the pilot treatment plant. Shown are the differences between the individual wastewater streams compared to the conventional treatment and the respective media controls. Significant effects were obtained using the Fisher LSD test, alpha = 0.05.

investigated wastewater stream	1st *L. variegatus* test		2nd *L. variegatus* test		*D. magna*		*L. minor*	
	abundance	biomass	abundance	biomass	repro-duction	population growth rate	growth rate	yield
B	↓	-	↓	-	-	↑	-	↓
B+O_3	↓	↓	-	↓	-	-	↓	↓
GAC	↓	↓	-	↓	-	-	↓	↓
GAC+O_2	↓	↓	-	↓	-	↑	↓	↓
BF	↓	↓	↓	↓	-	-	-	-
BF+O_2	↓	↓	↓	↓	↑	↑	↓	↓
MBR1	↓	↓	-	↓	↓	-	↓	↓
MBR1+O_3	↓	↓	-	↓	↓	-	-	-
MBR2	↓	↓	↑	-	↓	-	↓	↓

B = conventional treatment; B+O_3 = B after treatment with ozone; GAK = B+O_3 after activated carbon filtration; GAK+O_2 = B+O_3 after activated carbon filtration, aerated; BF = B+O_3 after biofiltration; BF+O_2 = B+O_3 after biofiltration, aerated; MBR1 = effluent of membrane bioreactor 1; MBR1+O_3 = effluent of membrane bioreactor 1 after treatment with ozone; MBR2 = membrane bioreactor 2

performance significantly improved in comparison to the conventional treatment (B)
performance significantly impaired in comparison to the conventional treatment (B)
performance not significantly different in comparison to the conventional treatment (B)
↑ performance significantly improved in comparison to the respective medium control
↓ performance significantly impaired in comparison to the respective medium control
- performance not significantly different in comparison to the respective medium control

- Infection rate of *Pasteuria*-exposed daphnids was reduced after exposure to the investigated wastewater streams in comparison with the *Pasteuria*-control
- Overall performance of daphnids in *Pasteuria*-treatments were slightly better than in *Pasteuria*-free treatments

No direct evidence for the formation of toxic TPs due to the advanced wastewater treatments was found, at least not in concentrations high enough to cause measurable effects in the investigated test systems. In addition, investigated wastewater effluents did not lead to immunotoxic effects, measurable with the chosen test system.
Yet, interpretation of effects related to possible TPs were hindered due to some study-site specific conditions, such as toxicity of conventional treated wastewater and elevated levels of nitrite and ammonium. Furthermore, effects seem to be species-

specific, with *L. variegatus* here being more sensitive to the tested wastewater streams than *D. magna* and *L. minor*. That highlights the necessity of a test battery consisting of representatives of different taxonomic groups to cover different trophic levels, habitats (e.g. sediment, water phase) and sensitivities to individual mode of actions, to ensure a comprehensive evaluation of the tested matrix.

Annex I.III, Schlüter-Vorberg et al., submitted 08/2018

In the present study, the impact of an immunosuppressive environmental pollutant on an invertebrate host-parasite system was investigated to improve knowledge on immunotoxicity in invertebrates, and to further explore the potential of the chosen system as invertebrate *host resistance assay*. The main findings are characterized as follows:

- While *Pasteuria* challenge alone did not lead to a significantly reduced host survival in comparison to the placebo-control, survival of *D. magna* was synergistically affected by the combination of CsA and Pasteuria
- The sterilization rate in *Pasteuria*-exposed *D. magna* was significantly increased in the combined treatments (simultaneously exposed to CsA and *P. ramosa*) compared to the *Pasteuria*-control
- Both investigated endpoints survival and *Pasteuria*-induced sterilization rate seem to be more sensitive in detecting immunotoxic effects in the present study, compared to the endpoints reproduction and intrinsic rate of population growth

The present study provides clear evidence for a suppressed disease resistance in an environmentally stressed host. Enhanced virulence of the natural *D. magna* parasite *P. ramosa* due to co-exposure to the immunosuppressant CsA was expressed both in terms of reduced host survival and increased host infection rate in combined treatments.

2.2 Effects of wastewater ozonation

Besides the beneficial effects of ozonation as advanced wastewater treatment method, such as the effective removal of pharmaceuticals and other micropollutants (Klavarioti et al., 2009; Margot et al., 2013), there are some mechanisms known, that may impair the quality of wastewater after treatment with ozone:

Due to its strong disinfectant properties (Camel & Bermond, 1998; von Gunten, 2003b) ozonation is an effective tool to reduce bacterial load and diversity in wastewater effluents, however recent studies reported the relationship between ozonation and a shift to selected antibiotic-resistant bacteria (Lüddeke et al., 2015; Alexander et al., 2016). Additionally, ozonation has been shown to lead to the formation of assimilable organic carbon (AOC) and the related subsequent undesired bacterial regrowth in water bodies (Hammes et al., 2006). The bacterial regrowth is of great concern especially when there is a potentiation of potentially pathogenic bacteria, such as coliforms, *Legionella* spp. and *Pseudomonas* spp. (van der Kooij, 1992). Furthermore, ozonation may increase the bioavailability of metals, due to mobilization of metal ions from suspended organic matters and thus will lead to an increased toxicity. Gagnon et al. (2014) reported a significantly increased bioaccumulation of different investigated metals in the tissue of mussels after exposure to ozone-treated effluents.

Yet, the mechanism of greatest concern is the formation of largely unknown TPs, which is either influenced by the direct reaction of molecular ozone (O_3) with the compound, or by indirect reaction with HO radicals (Langlais et al., 1991; von Gunten, 2003a). Ozonation may result in an increase of the polarity and the number of functional groups of a compound, which can be both advantageous and disadvantageous. In the case of pharmaceuticals, it is assumed that the intended mode of action will mainly disappear, resulting in TPs that are less active and thus less toxic, as for example shown for hydroxylated estrogens and hydroxylated antibiotics (Ternes et al., 2003). However, if the active moiety remains unchanged after the transformation process, some TPs may retain the same mode of action (Boxall et al., 2004; Evgenidou et al., 2015). Transformation can also lead to the creation of new toxicophores (chemical structures that are related to the toxicity of a compound) and thus result in an increased toxicity by similar or altered mode of action (Michael et al., 2014).

2.3 Potential toxicity of TPs

In general, the occurrence of TPs in the environment is of great concern, since they may be more polar, more abundant and detectable in higher concentrations in investigated water bodies, compared to their parent compound (Kolpin et al., 2000; Boxall et al., 2004; Lapworth & Gooddy, 2006; Lapworth et al., 2012). Simultaneously it is assumed that the majority of all emerging TPs is most likely not yet identified (Escher & Fenner, 2011).

In a comprehensive study Boxall et al. (2004) compared acute toxicity data of a huge number of pesticides, biocides and their degradates for fish, daphnia and algae. In 80% of the investigated compounds, the TPs were less or as toxic as the parent compound, yet the remaining 20% exhibited a higher toxicity than the parent compound, with an up to 100-fold toxicity increase observed for some of the investigated compounds. Even biological treatment may be accompanied by a toxification of compounds, as demonstrated e.g. for the formation of the toxicologically more potent 4-chlorphenol from clofibric acid (Kosjek et al., 2009). Yet, especially the treatment with ozone is known to highly contribute to the formation of TPs with in some cases higher toxicity compared to the parent compound. This was for example demonstrated among others for clofibric acid, DEET, ibuprofen, metoprolol, paracetamol, and thiamethoxam (Rosal et al., 2009; Benitez et al., 2013; Hamdi El Najjar et al., 2014). Ozonation may lead to the formation of TPs with functional groups such as aldehydes or hydroxylated aromatic compounds, that are known to exhibit a higher toxicological potency, as for example demonstrated for ozonation of propranolol (Benner & Ternes, 2009a). Aldehydes in general may reveal carcinogenic or mutagenic potential, due to the ability to interact with DNA (Richardson et al., 2007; Benner & Ternes, 2009b). A carcinogenic or mutagenic potential of wastewater after ozonation processes was already demonstrated by the formation of NDMA, due to ozonation of the fungicide tolylfluanide (Schmidt & Brauch, 2008), or the formation of bromate in the presence of bromide (von Gunten, 2003b).

Toxicity investigations of TPs are severely limited by the non-availability of most TPs as pure substances for experimental testing (Evgenidou et al., 2015; Fatta-Kassinos et al., 2016). The ecotoxicological investigated TPs in the present thesis (Annex I.I), were therefore produced in lab-scale batch experiments, using treated wastewater and

sewage sludge incubated with ACV under aerobic conditions to generate C-ACV, with subsequent ozone-treatment to generate COFA.

The only change in the molecular structure during transformation of ACV to C-ACV is the formation of a carboxylic acid, while transformation of C-ACV to COFA leads to a considerably different chemical structure (Fig. 4). The possibility that these alterations are responsible for the observed toxicity of C-ACV to *Daphnia* and that of COFA to algae cannot be excluded, as even small structural changes may affect receptor-molecule interactions and thereby lead to an altered toxicity (Boxall 2004).

However, the toxicities observed in both tests are comparatively low, thus indicating an unspecific toxicity rather than a specific mode of action. The toxicity of COFA against *R. subcapitata* may furthermore be a toxicokinetic effect of ion trapping of the charged substance species inside the algal cells (Neuwoehner & Escher, 2011).

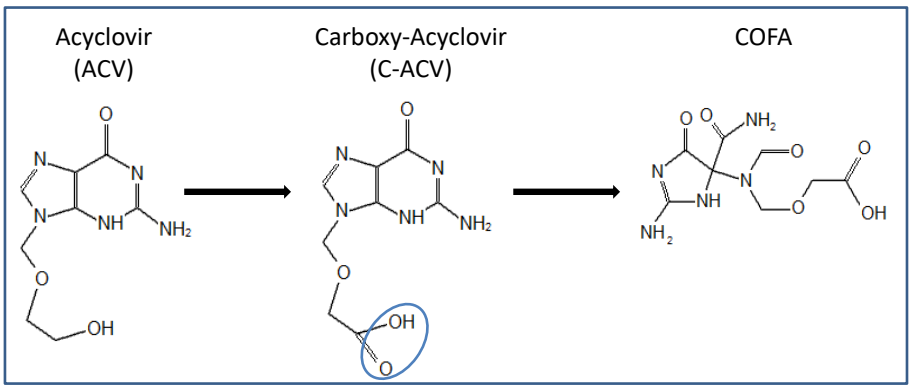

Figure 5: Molecular structure of ACV and two of its known transformation products C-ACV and COFA. The blue circle indicates the formation of the carboxylic acid in the molecular structure of C-ACV after oxidation of the primary hydroxycarboxylic acid of ACV.

The effective concentrations detected in the presented experiment are far higher than measured environmental concentrations, which are 2.4 µg/l and 0.001 µg/l for C-ACV and COFA respectively (Prasse et al., 2011; Knopp et al., 2016). Results therefore do not give direct cause for concern. However, this study provides evidence for the emergence of TPs with toxic potentials in the course of advanced wastewater treatment using the example of a pharmaceutical and its known TPs, that are actually detectable in surface and groundwaters.

TPs usually occur in complex mixtures, such as wastewater effluents, alongside their parent compounds. Thus, their contribution to the overall toxicity and the investigation of complex mixtures with appropriate test batteries should not be neglected.

2.4 The challenge of investigating complex mixtures/multiple stressors

Organisms in their natural habitats are generally exposed to a multitude of stressors, however ERA still focuses mainly on the investigation of individual stressors (Connon et al., 2012; Beyer et al., 2014). Unequivocally, these individual investigations are of great importance, as they provide important data on toxicity and dose-response relationships for single substances (Holmstrup et al., 2010). Yet, results of the standard toxicity testing are hard to transfer to the actual environmental situation, since many factors (such as bioavailability, interaction with other stressors and formation of TPs) influencing the mixture toxicity have to be considered (Heugens et al., 2001; Holmstrup et al., 2010; Laskowski et al., 2010). Additionally, even chemicals that are present in concentrations, individually causing no observable harm, nevertheless may contribute to mixture toxicity (Silva et al., 2002; Kortenkamp et al., 2009).

Effects of multiple stressors on affected organisms are hard to predict, even in relatively simple laboratory tests. The interaction of several factors can lead to a synergistic as well as an antagonistic interaction (Berenbaum, 1989; Folt et al., 1999; Cedergreen, 2014) and does not only seem to be substance-dependent, but also species-specific. Different models exist, trying to predict the toxicity of mixtures of chemicals. The concept of concentration addition (CA) for example, is valid for chemicals with known toxicity and exhibiting the same modes of action, whereas the model of response addition or independent action (IA) is applicable for chemicals acting specifically and dissimilar (Altenburger et al., 2004; Tang et al., 2013). CA has been recommended as precautionary first tier in environmental risk assessment of complex mixtures (Backhaus & Faust, 2012). Related to the investigation of wastewater samples, toxicity mixture concepts are only applicable for identified micropollutants in the complex mixture.

2.4.1 Ecotoxicological investigation of wastewater

Assessing the environmental risk of complex mixtures such as wastewater is challenging, due to the mostly unknown composition of the sample, and the mostly unknown ecotoxicity of most of the containing micropollutants (Beyer et al., 2014).
The assessment of chemical quality of wastewater effluents is currently mainly based on chemical analysis, with measurements of global parameters such as BOD, COD, TSS and TOC and selected organic trace substances according to the Urban Wastewater Treatment Directive 91/271/EEC. Despite continual improvement of analytical methods, current chemical target analysis is not able to identify all containing chemical components, and may fail to detect especially the unknown, however simultaneously potentially harmful compounds, as well as potential mixture toxicity (Eggen et al., 2004; Välitalo et al., 2017). Additionally, chemical analysis alone is not sufficient for the representation of biological responses caused by harmful components present in the wastewater effluents (Hernando et al., 2005). Therefore, the combination of chemical analysis with effect-based toxicity bioassays such as *in vitro* or in *vivo tests* with investigation of relevant endpoints, is of great importance for the complementary investigation of wastewater samples (Farré & Barceló, 2003; Connon et al., 2012).
In vitro assays are cell-based, and often receptor mediated assays, that react sensitively and specific to a certain mode of action (e.g. genotoxic or estrogenic effects). These assays are relatively easy to apply and thus provide a suitable method for fast and simultaneous screening of a bigger number of samples. However, the ecological relevance of results of *in vitro* tests is limited since the cell-based experiments rather uncover toxicological potentials instead of replicating the precise cellular response in intact organisms.
To observe the holistic toxicity on living organisms, the application of *in vivo* testing is therefore favorable. The most important advantage of *in vivo* biotests is the ability to capture effects of a broad range of the containing compounds with different mode of actions. Even effects of compounds not covered by the chemical analysis, such as TPs either entering the WWTP or being formed there during the treatment process are considered. A chosen test battery should therefore cover species of different habitats and different trophic levels, referring to different endpoints, to ideally depict the whole range of possible effects.

In recent years several studies were conducted investigating advanced treated wastewater, with focus on ozonated secondary effluents using vertebrate and invertebrate *in vivo* bioassays. In an acute toxicity test with bacteria (*Alliovibrio fischeri*) ozonated effluents were reported to cause toxic effects, which decreased with increasing sample storage time (Petala et al., 2006). In a study with embryos of *Oryzias latipes* (Japanese medaka) a significantly increasing 4-day mortality was observed with increasing ozone doses (Cao et al., 2009). Stalter et al. (2010a, b) and Magdeburg et al. (2012, 2014) observed increased toxicity of treated wastewater after half and full scale ozonation on *L. variegatus* and rainbow trout (*Oncorhynchus mykiss),* pointing to the emergence of toxic TPs after ozonation. A significant toxicity decrease was observed after a subsequent sand filtration step, elucidating the importance of additional post treatment, such as biologically active filtering processes, for the removal of toxic byproducts. In feeding trials with the crustacean *Gammarus fossarum* and lab-scale ozonated wastewater, the authors observed a slight toxicity reduction after ozonation, but assumed the formation of toxic TPs, that eventually masked positive effects related to ozonation of the wastewater effluents (Bundschuh et al., 2011). Da Costa et al., 2014 reported an increased toxicity of domestic secondary effluents after lab-scale ozonation, investigated in acute toxicity tests with crustacean (*Ceriodaphnia silvestrii*, *Daphnia similis*) and fish (*Danio rerio*).

Besides the studies elucidating the disadvantages of ozonation as tertiary treatment step, leading to the formation of toxic TPs (as mentioned above), several studies were found reporting also the beneficial side of wastewater ozonation. Escher et al. (2009) observed an enhanced water quality after ozone treatment for *A. fischeri* exposed to eluates of ozone and non-ozone treated wastewater. Likewise, Bundschuh & Schulz (2011) observed a reduced toxicity to crustacean (*G. fossarum*) after exposure to full-scale ozonated wastewater of the same WWTP. Ozonation of primary-treated effluent of urban wastewater was reported to significantly decrease the toxic potency of pollutants to the freshwater mussel *Elliptio complanata* (Gagné et al., 2007). Cao et al. (2009) observed the elimination of acute toxicity of conventional treated wastewater towards *D. magna* due to ozone doses ranging from 4 to 15 mg/l. In a study with lab-scale ozonation of secondary effluents, a 50% immobilization rate of *D. magna* was observed due to exposure against secondary effluents. After exposure to ozonated

samples, toxicity was reduced, yet still moderately pronounced (Kontana et al., 2009). Lundström et al. (2010) reported improved effluent quality and decreased toxicity after advanced treatment, including ozonation, investigated with a bioassay test batterie including among others *Danio rerio* (zebra fish) and *Pseudokirchneriella subcapitata* (micro algae). Margot et al. (2013) observed a significantly decreased toxicity of WWTP effluents on algae and fish (*O. mykiss*) after full scale ozonation.

Within the present thesis the ecotoxicological investigation of differently treated wastewater streams was realized applying an *in vivo* test battery including *L. variegatus*, *D. magna* and *L. minor* (Annex I.II). The experiments were carried out in flow through systems investigating non-stored effluents under real conditions, covering temporary fluctuations in the wastewater composition over the entire test duration. In contrast to expectations and in concordance with studies mentioned above, reporting quality enhancement after ozonation of wastewater, the present study detected an improved performance of *L. variegatus* after ozonation in comparison to treatments exposed to conventional treated wastewater. However, some study-site specific conditions have to be considered, that eventually hindered the identification and interpretation of toxic effects related to TPs, that were evidenced by the associated chemical analysis (Knopp et al., 2016). As mentioned before, the conventional treated wastewater, which served as influent for the advanced treatment processes, assumable still contained amounts of (micro)pollutants in concentrations high enough to significantly decrease abundance of *L. variegatus* compared to the medium control. Since these negative effects were relativized after subsequent ozone and filtering treatments, potential negative effects due to the process of ozonation (with potential formation of toxic TPs) were eventually masked. Simultaneously elevated levels of nitrite and ammonia were observed in the effluent of the MBR1 before and after ozonation, resulting in high mortality rates of *L. variegatus*. Ammonia and nitrite are highly toxic for aquatic invertebrate organisms (Epifanio & Srna, 1975; Schubaur-Berigan et al., 1995; Egeler et al., 2010; Romano & Zeng, 2013). Therefore, the distinction between toxic effects caused by elevated nutrient concentrations or by anthropogenic micropollutants was aggravated. Results gained from the tests with *D. magna* and *L. minor* also did not indicate the formation of toxic TPs, at least not in relevant concentrations causing toxic effects observable with the investigated biotests

and endpoints. As mentioned by Stalter et al. (2010) and confirmed by results of the present thesis, the growth inhibition test with *L. minor* appears to not be sensitive enough for the reliable detection of toxicity induced by micropollutants or TPs present in wastewater samples. Furthermore, *L. minor* seems vulnerable to nutrient removal, as for example given in GAK filtering systems, which additionally may hinder the interpretation of toxic effects due to pollutants. In contrast, *L. variegatus* exhibits a high sensitivity towards wastewater including potential toxic components, rendering it to be a promising organism for the *in vivo* investigation of (ozonated) wastewater (Stalter et al., 2010; Magdeburg et al., 2012).

Overall, the comparison of results of different studies investigating the complex wastewater matrix is complicated, and results may be contradictory due to highly diverse initial conditions regarding wastewater compositions, reactor configurations and applied biotests. A promising approach to assess the environmental impact of mixtures of micropollutants in WWTP effluents may be the use of a prospective quantitative ERA, as recently proposed by Coors et al. (2018). Yet, all relevant compounds along their concentrations have to be identified for the successful prospective quantitative ERA of WWTP effluents, which again aggravates the characterization of samples with an unknown composition.

2.4.2 Micropollutant-induced immunotoxicity in invertebrates

While many classes of environmental pollutants have been demonstrated to possess the ability to disrupt immune functions in humans and wildlife, such as heavy metals, polycyclic aromatic hydrocarbons, dioxins and pesticides (Fournier et al., 2000a; Vos, 2007), invertebrate immunotoxicity is still underrated in ecotoxicological risk assessment.

Immunotoxic substances may disturb the invertebrate immune system through a variety of mechanisms, including direct effects on components of the immune system, as well as indirect activation of stress response mechanisms and may result in an enhanced susceptibility to infectious agents (Fournier et al., 2000b; Descotes, 2004). Immunotoxicity in invertebrates can be detected with help of biomarkers, which are defined as measurements of biological responses in body fluids, tissue samples or at

the whole organism level (Depledge & Fossi, 1994). The investigation of immune biomarkers is an effective tool to estimate an immunotoxic potential of a substance. Yet, changes in these individual cellular immune functions do not necessarily translate into organismal functions or an impaired overall immune response, with exception of mechanisms directly related to population growth rate, the reproductive output or the viability of offspring (Depledge & Fossi, 1994; Galloway & Depledge, 2001).

In order to confirm causal relationships between immunotoxicity and exposure to pollutants, an experimental approach with *in vivo* experiments is therefore indispensable. Here *host resistance assays* provide a useful measure of the overall effective immune response related to host immunity and susceptibility to infectious pathogens (Thomas & Sherwood, 1996; Descotes, 2006; Burleson & Burleson, 2008). *Host resistance assays* have already been recommended as routine tests following an observed immunosuppression identified for example in preceding biomarker assays (Fournier et al., 2000b), however to date still no standardized *host resistance assays* exist for representatives of invertebrate organisms.

The experiments conducted in the present thesis (Annex I.III) implied a valuable contribution to the further development of an invertebrate *host resistance assay* that provides reliable and causal evidence of immunotoxicity. Results of the laboratory tests with the applied model substance CsA delivered information on the immunotoxicity of an intentionally immunosuppressive pharmaceutical with investigation of the immune response on a non-target organism. Results additionally proved the applicability of the chosen host-parasite system *D. magna* & *P. ramosa*.

2.4.3 The immunotoxic potential of wastewater

The host-parasite model *D. magna* & *P. ramosa* was likewise applied on the matrix of wastewater to investigate potential immunotoxic effects of conventional and ozone-treated wastewater without and with subsequent filtration steps (Annex I.II).

Due to the mostly unknown composition of wastewater, the presence of potential immunotoxic components such as micropollutants and their metabolites, cannot be excluded even in treated wastewater. Especially aquatic organisms living in close proximity to WWTP effluents may be affected and potential effects of long-term exposure to environmental pollutants present in wastewater effluents require further attention (Blaise et al., 2002). In several studies, using mainly biomarkers, immunosuppressant effects of different wastewater effluents have already been proven. In experiments with caged mussels (*Elliptio complanata, Dreissena polymorpha*), exposed downstream of municipal WWTP effluents, significant stress responses, such as a decreased phagocytic activity and an increased number of hemocytes were observed (Blaise et al., 2002; Gagné et al., 2002). Likewise, a reduced phagocytic activity was observed in a laboratory test with mussels (*Mytilus edilus*) exposed to municipal effluents (Akaishi et al., 2007). Gagné et al. (2008) investigated the immunotoxic potential of wastewater before and after treatment with ozone in a laboratory flow-through test with the mussel *Elliptio complanata*. While significantly inhibited phagocytosis and reduced cell viability were observed after exposure to a primary treated effluent, ozonation was shown to reduce cytotoxicity but was not successful in the enhancement of phagocytic activity.

As summarized above, a substantial number of studies exists, that investigated immunotoxic effects of municipal effluents with help of biomarkers, while only two studies were found, that addressed the topic of potential immunotoxicity of wastewater by means of *host resistance assays*. In a study with *M. edulis*, mussels exposed to samples containing 100% wastewater and subsequently infected with the gram-negative bacterium *Vibrio anguillarium*, died 24 h after the bacterial challenge, whereas mortality in mussels exposed to diluted wastewater (30% v/v effluent) was only 30% (Akaishi et al., 2007). Francois et al. (2015) observed increased hemocyte counts and phagocytosis activity in mussels exposed to municipal effluent. The induced

inflammation responses were shown to be enhanced due to exposure to wastewater and the simultaneous challenge with *V. anguillarium*.

It is noticeable that in summarized studies only bivalves (mussels) were investigated. Motive therefore may be the potential of disease outbreaks in edible species, eventually living in WWTP influenced habitats, such as effluent receiving rivers or coastal waters. The edible mussel *M. edulis*, that lives in coastal waters as well as in estuaries, therefore belongs to the well-studied bivalves among the group of molluscs (Galloway & Depledge, 2001).

The study conducted in the present thesis (Annex I.II) is the first to assess effects of conventional and advanced treated wastewater on the immune status of *D. magna* as one of the ecotoxicological key species. In contrast to summarized studies, no indications of immunotoxic effects of the conventional treated wastewater as well as of the advanced treated effluents were detected in experiments conducted in the course of the present thesis. There was even a reduction in the *Pasteuria*-induced infection rate of *D. magna* which may be related to promoting effects of treated wastewater on *D. magna*. These promoting effects are probably mainly caused by suspended solids and bacteria present in the wastewater, which are optimally utilized by *D. magna* as filter feeder (Lampert, 1987) and thus may contribute to an improved fitness and hence to an improved resistance to the parasite challenge. Additionally, a disinfecting effect, due to the ozone-treatment and the presence of potential residues of ozone, resulting in a reduction of the activity of the parasite *P. ramosa* cannot be excluded. In studies with *Pasteuria*-infected *D. magna* it was shown that a present infection can be cured with antibiotics, such as tetracycline (Little & Ebert, 2000; Duneau et al., 2016). The definite concentration of tetracycline present in the investigated wastewaters was not measured, however the presence of antibacterial substances such as antibiotics in treated wastewater is highly likely (Michael et al., 2013).

Overall, the investigation of the immunotoxic potential of wastewater is a challenging task, due to the mostly unknown composition of the matrix, as well as the heterogenous and highly species-specific immune responses of invertebrate species. This combination aggravates predictions and the transfer of results from one species to another, highlighting again the need of the development of standardized test systems.

2.5 Conclusion

- The present thesis provides evidence for the toxification of wastewater in the course of advanced wastewater treatment, due to the formation of TPs, in particular in the case of ozonation. It demonstrates that emerging TPs may exhibit greater aquatic toxicity than their parent compounds. Results highlight the importance of further research related to the topic of toxicity of TPs, both in terms of identification as well as toxicity characterization of TPs.

- The present thesis demonstrates that (A) biologically treated wastewater can have negative effects on chronically exposed organisms, that (B) advanced treatment of biologically treated wastewater can improve water quality for respective organisms, and (C) ozonation and the associated formation of transformation products proven by chemical analysis, does not necessarily lead to measurable negative effects on chronically exposed organisms.

- Results of the present thesis provides clear evidence for a suppressed disease resistance in an environmentally stressed host, highlighting the need of considering relevant factors such as the conjunction of environmental pollutants and pathogens in the current risk assessment of anthropogenic pollutants. Standard toxicity testing as employed in the current way of conducting ecological risk assessments for anthropogenic substances does not consider natural antagonists such as infectious diseases, and thereby likely underestimates the impact these substances may pose to natural populations in the environment.

Overall, species-specific toxicity of co-occurring compounds in complex mixtures may translate to unexpected effects at the ecosystem level, which highlights the importance of applying a test battery covering different taxonomic and trophic levels to reliably characterize the ecotoxicological potential of the investigated matrices. Additionally, the selection of relevant endpoints to be investigated, is enormously important in this context in order to avoid unilateral and possibly false-positive or false-negative results and to detect among others also immunotoxic potentials.

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Annex

I Publications and manuscripts as part of the thesis

I.I Toxification by Transformation in Conventional and Advanced Wastewater Treatment: The Antiviral Drug Acyclovir

Schlüter-Vorberg, L., Prasse, C., Ternes, T.A., Mückter, H., Coors, A., 2015.
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Authors contributions:

Initials of participating authors:

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Development and planning:

LS: 50%

AC: 30%

CP: 20%

Experimental phase:

LS: 75%; Conducting of ecotoxicity experiments

CP: 20%; Generation of transformation products and chemical analysis

HM: 5%; Conducting of genotoxicity experiments

Collecting of data and preparation of figures and tables:

LS: 90%; Collecting of data and preparation of figures and tables regarding ecotoxicity experiments

HM: 10%; Collecting of data and preparation of figures and tables regarding genotoxicity experiments

Analysis of data:

LS: 70%; Statistical analysis and interpretation of data regarding ecotoxicity experiments

AC: 20%; Support with statistic-expertise

HM: 10%; Statistical analysis and interpretation of data regarding genotoxicity experiments

Drafting of the manuscript:

LS: 70%; Writing of the manuscript

CP: 10%; Writing of the manuscript; Correction and revision

AC, HM, TT: 20%; Correction and revision

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Toxification by Transformation in Conventional and Advanced Wastewater Treatment: The Antiviral Drug Acyclovir

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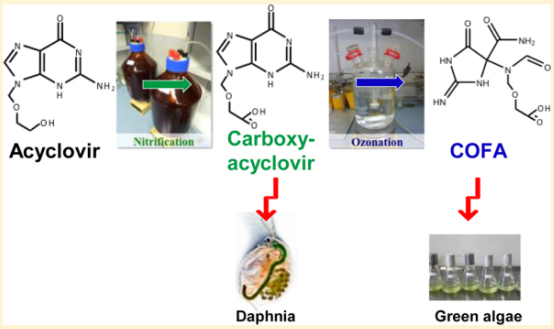
Supporting Information

ABSTRACT: Ozonation applied for advanced (waste)water treatment has a great potential to form polar transformation products (TPs) with often unknown toxicity. The antiviral drug acyclovir is transformed during biological wastewater treatment into carboxy-acyclovir. Ozone further transforms carboxy-acyclovir into *N*-(4-carbamoyl-2-imino-5-oxoimidazolidin)formamido-*n*-methoxy-acid (COFA). Both TPs have been detected in environmental samples and finished drinking water. Here, carboxy-acyclovir and COFA were produced at bench scale using treated wastewater and sewage sludge and were tested for aquatic toxicity in parallel with acyclovir. Carboxy-acyclovir was found to significantly reduce the level of reproduction of *Daphnia magna* (by 40% at 102 mg L^{-1}), and COFA inhibited the growth of green algae (E_rC_{10} of 14.1 mg L^{-1}); no toxicity was observed for acyclovir up to 100 mg L^{-1}. The predicted genotoxicity was not increased compared to that of the parent compound. In summary, the results highlight the importance of assessing the ecotoxicity of TPs formed during wastewater treatment, particularly in the case of ozonation.



INTRODUCTION

While knowledge of the environmental fate and effects of pharmaceuticals has improved considerably in recent years, similar information for their biotic and abiotic transformation products (TPs) formed naturally and in technical (waste)water treatment processes is widely lacking.[1−5] Whereas TPs are often reported to be less toxic than their parent compounds,[2,6−8] a study dealing with only pesticides and biocides indicated that in 20% of the cases the TPs exhibited an acute aquatic toxicity at least 3 times greater than that of the respective parent.[8] It remains unknown whether this finding can be transferred to the aquatic toxicity of TPs formed from other organic compounds such as pharmaceuticals. TPs that are formed in relevant amounts in the environment or by metabolic processes often have to be considered in the regulatory environmental risk assessment of the respective parent compound. In contrast, knowledge of the identity and potential hazard of TPs formed in conventional and advanced wastewater treatment processes is often not a standard requirement in regulatory environmental risk assessments. In particular, oxidation processes such as ozonation are known to be highly efficient with regard to primary degradation of a broad range of organic substances but may result in the formation of a great number of stable TPs with often unknown identity and toxicity[1,9−12] and genotoxic potential because of an increased reactivity.[13,14] The antiviral drug acyclovir (ACV), of which 45−75% is excreted by patients as unchanged compound,[15−17] is an example of a pharmaceutical with structurally identified TPs that are produced in wastewater treatment processes. Carboxy-acyclovir (C-ACV) is formed from ACV during nitrification and is transformed into *N*-(4-carbamoyl-2-imino-5-oxoimidazolidin)formamido-*n*-methoxy-acid (COFA) by ozonation. Because of its biological stability and high polarity, COFA cannot be removed by sand or activated carbon filtration. Both TPs have been detected in German river waters, wastewater treatment plant (WWTP) influents, effluents, and also finished drinking water.[18−20] The detection of ACV and its TPs in a broad range of environmental samples emphasizes the importance of the identification of TPs in the aquatic environment and in drinking water[2] and highlights the relevance of assessing (eco-)toxicological effects of these specific TPs.

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This study aimed to assess the aquatic ecotoxicity and to predict the genotoxic potential of ACV, C-ACV, and COFA. Sufficient quantities of TPs are prohibitively costly to synthesize, which often hinders their ecotoxicological testing. Here, the TPs were produced at laboratory scale using a setup in which treated wastewater and sewage sludge were incubated under aerobic conditions with ACV and ozonated thereafter. The whole laboratory treatment process was run in parallel without the addition of ACV to obtain controls that allowed separation of the effects of the TPs from effects of the treatments. Growth inhibition in green algae (*Raphidocelis subcapitata*), inhibition of the reproduction of the crustacean *Daphnia magna*, and survival of zebrafish embryos (*Danio rerio*) were used to assess aquatic toxicity at different trophic levels. Genotoxic potentials of ACV and its TPs were evaluated using the Distributed Structure-Searchable Toxicity (DSSTox) Database Network[21] and lazar (lazy structure−activity relationship) as the front end.[22]

METHODS

Biodegradation and Ozonation Experiments and Process Control Treatments. Biotransformation of ACV was achieved in two 10 L laboratory batch reactors. Sewage sludge from a nitrification unit of a German WWTP was diluted with treated effluent and continuously stirred and aerated with a mixture of air and CO_2 to maintain aerobic conditions and a stable pH of 7 ± 0.2. A freshly prepared stock solution of ACV dissolved in treated effluent was added, resulting in a final concentration of 200 mg L^{-1}. After complete transformation of ACV, the slurry was filtered and 2 L aliquots of the filtrate were subsequently ozonated. Biotransformation of ACV and oxidative transformation of C-ACV during ozonation were monitored using liquid chromatography−tandem mass spectrometry (LC−MS/MS).[19] The same setup was used for the process controls without adding ACV. Aliquot samples of all treatments were stored frozen (−20 °C) prior to testing in the different biotests. Further details can be found in the Supporting Information.

The terms C-ACV and COFA are used in the following to denote the treatments in which C-ACV and COFA were produced. The respective process controls are labeled as B (biological treatment) and B+O (biological treatment followed by ozonation).

Biotests. The following treatments were tested in parallel in each biotest: ACV, C-ACV, COFA, process control treatments B and B+O, and control treatment C0 consisting of culture medium of the respective test species. The parent compound ACV (CAS Registry Number 59277-89-3, Sigma-Aldrich, 99.6% pure) was dissolved directly in respective culture media and tested at 100 mg L^{-1}. Samples of C-ACV, COFA, B, and B+O were diluted with an equal amount of 2-fold concentrated culture medium of the respective biotest to ensure sufficient nutrient content for the test organisms. For detailed information about the biotests and used culture media, see the Supporting Information.

Algal Growth Inhibition Test. A static 72 h algal growth inhibition test was conducted with *Raphidocelis subcapitata* according to OECD 201. A second test was conducted in an identical way with a geometric dilution series (eight concentration levels with a spacing factor of 1.8) of the COFA treatment. The response variables biomass yield and growth rate after 72 h were evaluated for both tests.

***D. magna* Reproduction Test.** A semistatic 21 day reproduction test was conducted with *D. magna* according to OECD 211. The response variables survival, number of living offspring per surviving female within 21 days, and intrinsic rate of population growth were evaluated.

***D. rerio* Embryo Toxicity Test.** A static 96 h embryo toxicity test was conducted with embryos of in-house cultured zebrafish (*D. rerio*) according to OECD 236. The resulting response variable survival after 96 h was evaluated.

Analytical Measurements. Test solutions were sampled every week during the *Daphnia* test from corresponding fresh and aged media of every treatment and at the beginning and end of the first algal test. All samples were stored frozen at −20 °C until they were analyzed. ACV and C-ACV were analyzed using LC−MS/MS.[19] Concentrations of COFA were determined by the standard addition method using five spiking levels.

Statistical Analysis. Compliance with the assumptions of normal error distribution and homogeneous variances were confirmed visually and by Bartlett's, Cochran's, and Hartley's tests (at $\alpha = 0.01$), for the response variables algal yield, algal growth rate, *Daphnia* offspring, and *Daphnia* growth rate. Subsequently, a Tukey HSD test was performed in STATISTICA (version 12) to test for significant differences (two-sided, $\alpha = 0.05$) between treatments.

Using the software R and the *drc* package,[23] results for the response variables yield and growth rate determined in the second algal test were related to analytical measured concentrations of COFA and fitted by a three-parameter log−logistic model to estimate concentrations with 10 and 50% effects (EC_{10} and EC_{50}, respectively).

Genotoxicity Prediction. In the absence of valid data or sufficient amounts of substance for experimental testing, it is common practice to explore toxicological databases, expert systems, and other *in silico* approaches to assess the toxic potential of the chemicals of interest.[24] Here estimates of the genotoxic potential of ACV, C-ACV, and COFA were obtained with the help of the Distributed Structure-Searchable Toxicity (DSSTox) Database Network[21] via the lazar web interface. SMILES codes were generated from two-dimensional structures and inserted into the query form. The output provided qualitative estimates of the mutagenicity and carcinogenicity for mouse, rat, and hamster for the input structures. The decision was based on a fragment analysis and structure−activity-related comparisons.

RESULTS AND DISCUSSION

Biodegradation of ACV in the laboratory batch reactor was completed within 3 days, and the yield of C-ACV (approximately 200 mg L^{-1}) confirms complete transformation (molar mass balance of 106%). This is in good agreement with previous work showing that C-ACV is the only TP formed from ACV during biodegradation under aerobic conditions.[19] During ozonation, C-ACV was completely removed within 15 min and the final COFA concentration reached approximately 160 mg L^{-1}, demonstrating an incomplete transformation of 72% based on a molar mass balance, which was confirmed in measurements of biotest samples (Table 1), indicating the potential formation of other unidentified TP(s).

The analysis of C-ACV and COFA in the biotest samples confirmed the concentrations of TPs measured during the batch reaction. The concentrations measured in freshly prepared (initial) biotest solutions and those after exposure

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Table 1. Concentrations of ACV, C-ACV, and COFA in Samples of Biotest Treatments (ACV, dissolved in test medium; C-ACV, COFA, B, and B+O, wastewater samples of batch reactor treatments diluted 1:1 with test medium; control, test medium) at Test Start ($C_{initial}$) and after Exposure for 2−3 Days (C_{aged}) Given as Means (±standard deviation) of Measurements in the Algal and *Daphnia* Tests (n = 5 per sample)

sample	ACV (mg L^{-1})	C-ACV (mg L^{-1})	COFA (mg L^{-1})
$ACV_{initial}$	92.1 ± 6.5	<LOQ	<LOQ
ACV_{aged}	91.1 ± 9.0	<LOQ	<LOQ
$C\text{-}ACV_{initial}$	<LOQ	101.9 ± 14.1	0.2 ± 0.01
$C\text{-}ACV_{aged}$	<LOQ	105.3 ± 4.0	0.2 ± 0.02
$COFA_{initial}$	<LOQ	<LOQ	80.7 ± 3.0
$COFA_{aged}$	<LOQ	<LOQ	79.0 ± 5.6
B	<LOQ	0.001	<LOQ
B+O	<LOQ	<LOQ	<LOQ
control	<LOQ	<LOQ	<LOQ
LOQ	0.0001	0.0001	0.001

for 2−3 days (aged) confirmed that ACV and both TPs were stable during exposure (Table 1). ACV and COFA treatments contained neither of the two other analytes above their quantification limits, while the C-ACV treatment contained a small amount of COFA (<1%). Hence, effects observed in the biotests can be attributed directly to the presence of the individual TP (C-ACV or COFA) as long as statistically significant differences from the respective process control (B or B+O) are detected. Effects due to the presence of other chemicals or TPs can be identified by comparing the process and medium controls with each other.

The biotests fulfilled all validity criteria regarding water quality parameters (reported in the Supporting Information) and biological end points according to respective OECD guidelines. The only exception was the *D. rerio* embryo test, in which the hatching rate after 96 h was only 17% in the laboratory control instead of the required 80%. However, embryo survival after 96 h reached at least 95% in all wastewater and control treatments, and the required reduction of survival (20%) was achieved in the positive control. No sublethal effects were observed, indicating no acute fish toxicity of ACV and both TPs up to a concentration of ∼100 mg L^{-1}.

No mortality was observed in the *Daphnia* reproduction test. Reproduction and population growth rate of *D. magna* did not differ between the medium control and the ACV treatment (Figure 1), demonstrating that ACV exhibits no chronic *Daphnia* toxicity up to a concentration of 92.1 mg L^{-1}. Reproduction and population growth rate were significantly enhanced in the process control of the biological treatment (B) as well as in that of the biological treatment followed by ozonation (B+O) compared to the medium control. This effect may be attributed to better food conditions resulting from the bacterial load provided by the biological treatment. Reproduction and population growth rate were significantly reduced in the C-ACV treatment compared to the respective process control treatment B (by 39.9 and 22.4%, respectively) and the laboratory control. This indicates a significant increase in *Daphnia* toxicity of C-ACV compared to that of the parent. No significant differences occurred between COFA and the B+O treatment, indicating that COFA was not toxic to *Daphnia*.

Algal yield and growth rate were significantly inhibited in the COFA treatment compared to all other treatments, which did

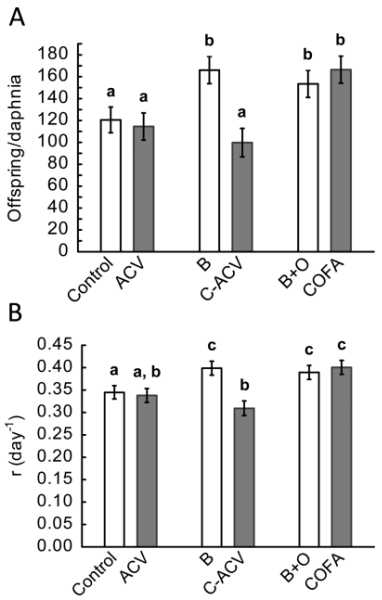

Figure 1. Reproduction measured as living offspring per female within 21 days (A) and intrinsic rate of population increase per day, r (B), of *D. magna* exposed to ACV, its transformation products present in the C-ACV and COFA treatments, the respective process control treatments B and B+O, and the medium control (M4). Shown are means with their 95% confidence intervals (n = 4 per treatment). Identical letters denote treatments that did not significantly differ from each other (Tukey HSD test; α = 0.05).

not differ among each other or from the (process) controls (Figure 2A,B). The toxicity of COFA toward algae was confirmed in the second test where an inhibition of yield and growth rate by 91.4 and 43.9%, respectively, was observed at the highest tested COFA concentration compared to the process control treatment B+O (Figure 2C,D). The EC_{10} (95% confidence interval) of COFA was estimated to be 4.12 (2.48−5.77) and 14.11 (11.17−17.06) mg L^{-1} for yield and growth rate, respectively. The EC_{50} was estimated to be 18.15 (15.44−20.87) and 101.57 (90.96−112.19) mg L^{-1} for yield and growth rate, respectively.

Effects of biologically active substances may be caused by specific receptor ligand interactions because even minor molecular modification of the active moiety of the molecule may lead to an altered toxicity in comparison with that of the parent compound.[8] The only structural alteration occurring due to the transformation of ACV to C-ACV is the formation of a carboxylic acid, while the transformation of C-ACV to COFA (see the graphical abstract) leads to a considerably different chemical structure. The possibility that these alterations are responsible for the observed toxicity of C-ACV to *Daphnia* and that of COFA to algae cannot be excluded. However, the observed toxicities in both cases are comparatively low, indicating an unspecific toxicity rather than a specific mode of action. The toxicity of COFA against *R. subcapitata* may furthermore be a toxicokinetic effect of ion trapping of the charged substance species inside the algal cells.[25]

Because of the incomplete mass balance for COFA, the possibility that other unidentified, minor TP(s) were formed from C-ACV during ozonation, which contributed to the observed algal toxicity, cannot be excluded. If we speculate that one other unidentified TP (produced at the remaining 28% of

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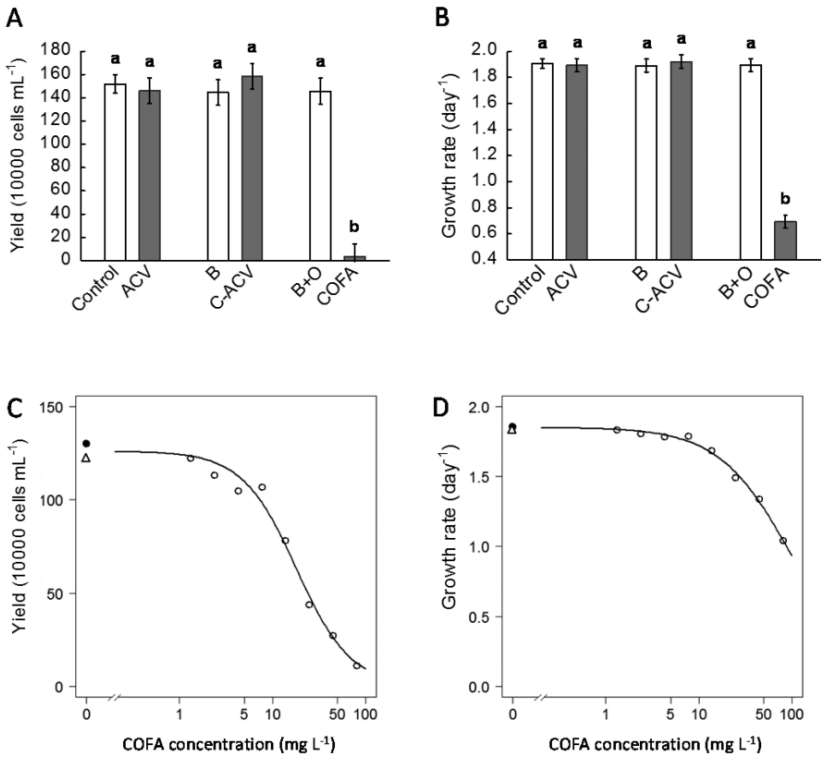

Figure 2. (A) Yield and (B) growth rate of *Raphidocelis subcapitata* after exposure for 72 h to ACV, its transformation products in the C-ACV and COFA treatments, the respective process control treatments B and B+O, and the control medium. Shown are mean responses with their 95% confidence intervals. Identical letters denote treatments that did not significantly differ from each other (Tukey HSD test; $\alpha = 0.05$). Concentration–response curves for (C) yield and (D) growth rate of *R. subcapitata* after exposure for 72 h to dilutions of the COFA treatment (based on measured COFA concentrations). Shown are means per treatment fitted by a three-parameter log–logistic model: (△) laboratory control, (●) process control treatment (B+O), and (○) COFA treatments.

the mass balance) fully accounted for the observed algal toxicity, this TP must have had a toxicity considerably higher than that calculated here for COFA. Because no other TPs could be identified during ozonation of C-ACV using very sensitive analytical techniques,[20] it appears most likely that either several TPs were formed at low concentrations or that the mass balance could not be closed because of limitations of the analytical methods (e.g., purity of the analytical standard).

The *in silico* predictions comprising reviewed mutagenicity and carcinogenicity data for common laboratory mammalians and *in vitro* test systems (see the Supporting Information, Table S5) did not suggest, on the basis of present structural evidence, a genotoxic potential of C-ACV and COFA greater than that of the parent compound ACV. Hence, no toxification regarding these end points by transformation was observed, which reduces the concern that ozonation of C-ACV-containing waters and subsequent movement of COFA to drinking water resources poses a risk to human health.

With the successful laboratory batch-scale production and ecotoxicological testing of C-ACV and COFA, the study presented here demonstrates a suitable approach to assessing the ecotoxicity of TPs that are not commercially available in sufficient amounts and quality. While the observed toxicity at 100 mg of C-ACV L^{-1} toward *D. magna* and the relevant toxicity estimate of COFA (14.11 mg L^{-1}, EC_{10} of algal growth rate inhibition) do not indicate an unacceptable environmental risk when compared with measured environmental concentrations of ~2.4 μg L^{-1}[19] and 0.001 μg L^{-1},[26] respectively, the results underline the general importance of studying the toxicity of TPs, even if they are formed from parent compounds showing no aquatic toxicity such as ACV. Similar to some TPs of pesticides and biocides,[8] TPs of pharmaceuticals can be more toxic than their parent compound. Species-specific toxicity of co-occurring compounds may translate to unexpected effects at the ecosystem level, which highlights the importance of applying a test battery covering different taxonomic and trophic levels to reliably characterize the ecotoxicological potential of TPs.

The TP(s) formed during ozonation (most likely COFA) exhibited the greatest increase in toxicity, which confirms the previous concern about the potential of oxidation processes such as ozonation to produce toxic TPs.[27] While the degree of toxicity increase observed for COFA (a factor of at least 7 more toxic to algae than its pharmacologically active parent compound; no increase in genotoxic potential) does not render ozonation *per se* as unsuitable for final wastewater purification, this study serves as an example providing clear evidence and should be an alert to the potential negative effects of ozonation for the receiving environment as many other wastewater-born chemicals may similarly form TPs of greater toxicity during the treatment process.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.5b00291.

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Detailed information about biodegradation and ozonation experiments, methods of conducted biotests, prediction of genotoxicity, and details of statistical analysis (PDF)

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Notes

The authors declare no competing financial interest.

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Supporting Information (SI)

to

Toxification by transformation in conventional and advanced wastewater treatment: the antiviral drug acyclovir

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Biodegradation and ozonation experiments and process control treatments

Biodegradation was achieved in two 10 L batch reactors. The sewage sludge was diluted 1:5 with treated effluent. In order to ensure complete dissolution before the start of the experiments (aqueous solubility: 1.3 g L^{-1})[1], acyclovir was dissolved in 2 L of wastewater effluent and the solution was ultrasonicated. After filtration (Whatman GF6 glassfiber filters), aliquots (2 L) of the biological treated wastewater were subsequently ozonated by directing the gaseous O_3 stream from the ozone generator directly into the batch reactor. The solution was stirred continuously to ensure homogeneous mixing. Measurements of pH prior and after ozonation confirmed stable conditions (pH 7 ± 0.4) during the experiments. Quantification of COFA was performed by standard addition using five different spiking levels.

Algae growth inhibition tests

Algae (*Raphidocelis subcapitata,* SAG 61.81, formerly *Pseudokirchneriella subcapita)* were inoculated with an initial density of 0.5 x 10^4 cells mL^{-1} from an exponentially growing pre-culture and statically exposed in Erlenmeyer flasks (100 mL test solution volume) at 21.6 - 23.0 °C (mean: 22.4 °C) and continuous light (85 - 91 mE/m^2 s^{-1}) for 72 h. The medium control (modified algae medium, OECD 201[2]) was run with six, all other treatments with three replicates. Fluorescence was measured every 24 h in aliquots of the test solutions using a spectral fluorometer (Tecan multiplate reader, Tecan Group) and converted to cell density based on a calibration curve prepared from the pre-culture. Results of measurements of pH at the beginning and the end of the first algae test are given in Table S1.

Table S1: Results of measurements of pH at the beginning and the end of the first test with *Raphidocelis subcapitata*, n = 2 per treatment.

Treatment	pH
ACV	7.7, 8.6
C-ACV	7.9, 9.1
COFA	7.9, 8.2
B	8.0, 9.6
B+O	8.0, 9.5
Control	8.0, 9.4

Medium control and B+O treatment in the second algae test were run with six, all COFA treatments with three replicates. Algae were exposed at 22.8 - 23.2 °C (mean: 22.9 °C) with continuous light of 82 - 90 mE/m^2 s^{-1}. Results of measurements of pH at the beginning and the end of the second algae test are given in Table S2.

Table S2: Results of measurements of pH at the beginning and the end of the second test with *Raphidocelis subcapitata*, n = 2 per treatment.

Treatment	pH
COFA C1	7.7, 9.3
COFA C2	7.8, 9.5
COFA C3	7.8, 9.0
COFA C4	7.8, 9.0
COFA C5	7.8, 8.3
COFA C6	7.8, 8.5
COFA C7	7.8, 8.2
COFA C8	7.8, 8.1
B+O	8.0, 8.5
Control	7.7, 8.5

Daphnia magna reproduction test

A *Daphnia magna* reproduction test was conducted according to OECD 211.[3] The *Daphnia magna* clone M10 used in the present study was obtained by KU Leuven, Belgium in 2011 and since then cultured as a clonal linage under optimal laboratory conditions. Stock culturing of *D. magna* was performed in Elendt medium M4[4] at a constant temperature of 20 °C +/− 2 °C under diffuse light with an intensity ranging from 50 – 1000 lx and a light/dark cycle of 16/8h. Green algae (*Desmodesmus subspicatus*), Tetramin and yeast were provided as food source in defined feeding intervals. Elendt medium M4 was also used for the laboratory control. Juvenile daphnids (<24h old) were individually exposed to 50 mL test solution. Exposure media were renewed three times per week, survival of test animals was checked and offspring were counted and removed from test beakers daily. Exposure was carried out with 10 replicates per treatment for 21 days at 19.7 - 21.1 °C (mean: 20.5 °C) under diffuse light (about 300 lx) and a light/dark cycle of 16/8h. Daphnids were fed green algae suspension (*Desmodesmus subspicatus*) three times per week with an amount equal to 0.1 mg C/daphnia/day from day 0 - 3 and 0.2 mg C/daphnia/day from day 4 until test end. Physical-chemical parameters measured during the exposure period are demonstrated in Table S3.

Table S3: Results of measurements of pH, oxygen and total hardness during the test with *Daphnia magna*, mean (min-max), n = 8 per treatment.

Treatment	pH	Oxygen		Total hardness
		[%]	[mg L^{-1}]	[°d]
ACV	7.9 (7.7-8.1)	92 (84-99)	8.2 (7.4-8.8)	16.2 (15.8-17.2)
C-ACV	8.1 (7.8-8.3)	95 (82-100)	8.4 (7.2-8.8)	16.1 (15.0-17.4)
COFA	8.1 (7.8-8.5)	95 (86-103)	8.4 (7.6-9.1)	17.2 (16.8-17.6)
B	8.2 (7.9-9.3)	94 (80-100)	8.3 (7.1-8.8)	16.9 (16.6-17.2)
B+O	8.1 (7.7-8.4)	94 (84-102)	8.3 (7.4-9.0)	17.2 (17.0-17.4)
Control	7.9 (7.5-8.2)	93 (85-99)	8.2 (7.5-8.8)	14.4 (14.0-14.8)

Danio rerio embryo toxicity test

Exposure of *Danio rerio* eggs started within one hour after fertilization and was performed in 24-well plates using 20 fertilized eggs per treatment introduced individually per well. Laboratory control treatment (reconstituted water according to OECD 203[5]) was run with 24 fertilized eggs on a separate plate plus four eggs per treatment as internal plate controls resulting in 48 control eggs. 3.4-dichloraniline (CAS 95-76-1; 3.7 mg/L; Sigma-Aldrich) was used as positive control.
Static exposure was conducted at 25.4 - 26.2 °C (mean: 25.8 °C) with light conditions of approximately 300 lx and a light:dark cycle of 12:12 h. Measurements of the physical-chemical parameters at the beginning and the end of the test with *Danio rerio* embryos are given in Table S4.
Lethal effects were evaluated according to OECD 236[6]: coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heartbeat. Lethal effects were checked every 24 h.

Table S4: Results of measurements of pH, oxygen, total hardness and conductivity at the beginning and the end of the test with *Danio rerio* embryos, n = 2 per treatment.

Treatment	pH	Oxygen		Total hardness	Conductivity
		[%]	[mg L^{-1}]	[°d]	[µS cm^{-1}]
ACV	7.0, 7.9	92, 94	7.6, 7.8	10.8, 12.0	856, 863
C-ACV	7.9, 8.0	93, 94	7.6, 7.9	18.0, 18.0	1041, 1068
COFA	8.0, 8.1	92, 92	7.4, 7.8	17.0, 17.2	936, 975
B	7.9, 8.0	93, 95	7.6, 7.9	18.0, 18.2	1072, 1075
B+O	8.0, 8.0	92, 94	7.6, 7.8	18.2, 18.6	1078, 1087
Control	6.6, 8.0	89, 94	7.6, 7.7	11.2, 11.2	834, 888
Positive control	6.7, 8.0	90, 95	7.6, 7.7	11.0, 11.2	852, 894

Statistical analysis
Results for the response variables yield and growth rate determined in the second algae test were fitted by a 3-parameter log-logistic model (LL.3) as:

$$f\,(x) = \frac{d}{1 + e^{b\,\log(x) - \log(e))}} \qquad \text{(eqn. 1)}$$

with d as upper limit (lower limit fixed at 0), *b* representing the steepness of the regression curve, and *e* being the median effect concentration (EC_{50}). EC_{50} and EC_{10} values and their confidence intervals (95%) were obtained with the implemented function “ED” of the *drc* package using the delta method and the t-distribution.

Genotoxicity prediction
C-ACV was devoid of any genotoxicity in the prediction model. COFA carries a similar predicted genotoxicity risk as ACV does (Table S5).

Table S5: *In-silico* prediction of the genotoxicity of ACV, C-ACV and COFA. Estimates were obtained by querying the DSSTox databases. "+" genotoxicity potential predicted, "-"no genotoxicity potential predicted.

In-silico approach	ACV	C-ACV	COFA
Single cell carcinogenicity	-	-	-
MultiCell	+	-	+
Mouse	+	-	+
Rat	-	-	-
Hamster	+	-	+
ISSCAN carcinogenicity	+	-	+
Kazius-Bursi mutagenicity	-	-	-

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I.II **Survival, reproduction, growth, and parasite resistance of aquatic organisms exposed on-site to wastewater treated by advanced treatment processes.**

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Development and planning of on-site *in vivo* tests:

LS: 70%

AC: 30%

Experimental phase:

LS: 100%; Conducting of on-site *in vivo* tests

Collecting of data and preparation of figures and tables:

LS: 90%; Collecting of data and preparation of figures and tables regarding results of on-site *in vivo* tests

GK: 10% Collecting and providing of data regarding chemical analysis

Analysis of data:

LS: 80%; Statistical analysis and interpretation of data regarding on-site *in vivo* tests

AC: 20%; Support with statistical expertise

Drafting of the manuscript:

LS: 70%; Writing of the manuscript

GK: 10%; Support with supplementary data regarding advanced wastewater treatment methods, physicochemical parameters and chemical analysis

AC, GK, PC, TT: 20%; Correction and revision

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Survival, reproduction, growth, and parasite resistance of aquatic organisms exposed on-site to wastewater treated by advanced treatment processes

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ABSTRACT

Advanced wastewater treatment technologies are generally known to be an effective tool for reducing micropollutant discharge into the aquatic environment. Nevertheless, some processes such as ozonation result in stable transformation products with often unknown toxicity. In the present study, whole effluents originating from nine different steps of advanced treatment combinations were compared for their aquatic toxicity. Assessed endpoints were survival, growth and reproduction of *Lumbriculus variegatus*, *Daphnia magna* and *Lemna minor* chronically exposed in on-site flow-through tests based on standard guidelines. The treatment combinations were activated sludge treatment followed by ozonation with subsequent filtration by granular activated carbon or biofilters and membrane bioreactor treatment of raw wastewater followed by ozonation. Additionally, the impact of treated wastewater on the immune response of invertebrates was investigated by challenging *D. magna* with a bacterial endoparasite. Conventionally treated wastewater reduced reproduction of *L. variegatus* by up to 46%, but did not affect *D. magna* and *L. minor* with regard to survival, growth, reproduction and parasite resistance. Instead, parasite susceptibility was significantly reduced in *D. magna* exposed to conventionally treated as well as ozonated wastewater in comparison to *D. magna* exposed to the medium control. None of the three test organisms provided clear evidence that wastewater ozonation leads to increased aquatic toxicity. Rather than to the presence of toxic transformation products, the affected performance of *L. variegatus* could be linked to elevated concentrations of ammonium and nitrite that likely resulted from treatment failures.

1. Introduction

Numerous chemicals, pharmaceuticals and personal care products (PPCP) are an indispensable part of everyday lives in industrialized countries. These substances are typically discharged into the sewer system and only partially removed in biological wastewater treatment processes. Wastewater treatment plants (WWTP) are therefore considered as main point source of PPCPs for the aquatic environment (Lapworth et al., 2012; Schwarzenbach et al., 2006; Ternes 1998). Since conventional wastewater treatment is optimized to remove nutrients and easily degradable organic compounds, recalcitrant organic micropollutants are eliminated to a lesser degree resulting in concentrations in the aquatic environment in the ng–μg L^{-1} range (Margot et al., 2013). Despite the very low environmental concentrations, these ubiquitous micropollutants may cause adverse effects on aquatic ecosystems (Ashauer 2016; Prasse et al., 2015; Santos et al., 2010; Liney et al., 2005). To reduce micropollutant discharge via effluents of WWTPs and improve water quality in aquatic ecosystems according to the European Water Framework Directive (EU-WFD) as well as based on precautionary principles, Switzerland currently pioneers in establishing advanced treatment methods on full-scale at 100 WWTP within the next 20 years (Eggen et al., 2014).

Advanced treatment processes such as ozonation, dosing powdered activated carbon (PAC) and granulated activated carbon (GAC) filtration have been extensively investigated for their potential to remove various selected micropollutants (Noguera-Oviedo and Aga, 2016; Hollender et al., 2009; Nowotny et al., 2007). Adsorption techniques using PAC or GAC or sand filter are particularly efficient in removing hydrophobic but not the more polar compounds from the water phase (Snyder et al., 2007; Ternes et al., 2004). Membrane bioreactors (MBRs) allow for higher sludge

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concentrations than conventional activated sludge treatment and retention of almost all suspended solids (Çiçek et al., 1999) and extended biological degradation can be achieved in biofilters that foster a biologically active biofilm on the filter material (Chaudhary et al., 2003). Oxidative techniques (such as ozonation) efficiently lead to degradation of a wide range of organic substances that are recalcitrant to biological degradation. However, the degradation can result in stable transformation products (TPs) (Bollmann et al., 2016), particularly at low doses of ozone (Schmidt and Brauch 2008). Although TPs are mostly less toxic than their parent compounds (Boxall et al., 2012; Sinclair and Boxall 2003), greater aquatic toxicity is known for some TPs of pesticides and biocides (Boxall et al., 2004). While knowledge on the toxicity of TPs of typical wastewater-born micropollutants such as PPCPs is still rare, a recent study documented elevated aquatic toxicity of the TPs of the antiviral drug acyclovir formed during wastewater treatment (Schlüter-Vorberg et al., 2015).

Whereas the removal efficiency of advanced treatment processes can be evaluated based on indicator substances (Jekel et al., 2015), only ecotoxicological tests enable determining the overall aquatic toxicity including that caused by micropollutants not covered by analytical measurements and unknown TPs (Vasquez and Fatta-Kassinos, 2013). Conventionally treated wastewater has been reported to exhibit negative effects on aquatic organisms after ozonation, possibly due to formation of toxic TPs (da Costa et al., 2014; Magdeburg et al., 2012; Stalter et al., 2010a).

The present study tested the hypothesis that advanced wastewater treatment methods or combinations thereof reduce or enhance chronic aquatic toxicity. Such changes may be due to elimination of micropollutants or formation of toxic transformation products. While *in vitro* bioassays or biomarkers may provide valuable information on potential mechanisms of toxicity of micropollutants and their TPs present in wastewater (Connon et al., 2012), the present study focused on assessing chronic toxicity at an integrative and population-relevant level by means of *in vivo* tests. The *in vivo* test battery consisted of a primary producer (*Lemna minor*), a pelagic (*Daphnia magna*) and a benthic (*Lumbriculus variegatus*) invertebrate as test organisms. The treatment combinations were ozonation followed by activated carbon- or biofiltration (Knopp et al., 2016) and MBR treatment (in comparison to conventional activated sludge treatment) with and without subsequent ozonation. The test organisms were chosen based on previous investigations indicating their susceptibility to ozonated wastewater (Magdeburg et al., 2012; Stalter et al., 2010b), and in order to cover different trophic levels and taxonomic groups. All tests were conducted in flow-through systems on-site at a pilot treatment plant, assessing whole effluents from all treatment processes in parallel. This has the advantage to cover peak and average concentrations of micropollutants and their TPs in chronic exposure scenarios. In addition to assessing effects on survival, growth and reproduction of the test organisms, the susceptibility of one test organism, *D. magna*, to parasites was investigated based on previous reports on the impact of wastewater on the immune system of invertebrates (Minguez et al., 2012; Chu 1999).

2. Material and methods

2.1. Characterization of WWTP, pilot treatment plant and wastewater effluents

A pilot treatment plant was established that employed various advanced processes to treat wastewater of municipal and industrial origin (about 70% and 30% respectively) received from a WWTP in Southern Hesse with population equivalents of about 42,000 and an annual average of discharge of 6400 $m^3\ d^{-1}$. This WWTP applies preliminary treatment with screening and grit removal (without primary settlement), secondary treatment designed as conventional activated sludge process implemented by activated sludge tanks with primary denitrification, nitrification and phosphorous removal by chemical precipitation and finally secondary clarification.

The nine advanced treatments at the pilot plant received feeds as summarized in Table 1.

The conventionally treated wastewater (B) was micro-filtrated (10 μm) prior to feeding the ozone system 1 which consisted of two bubble columns connected in series and one equalization tank. Unless stated otherwise, the process parameters are given as mean values ± standard deviation. The specific ozone consumption in the ozone system 1 was $0.9 \pm 0.2\ g_{O3}\ g_{DOC}^{-1}$ (n = 96) with a hydraulic retention time of around 18 min (n = 96) during the test period. Two GAC filters and two expanded clay biofilters were designed identically and operated in parallel. One GAC filter and one biofilter were aerated by ambient air, while the other two filters were run non-aerated. Pre-selected granular activated carbon (internal surface 1200 $m^2\ g^{-1}$, grain size 1–4.8 mm) and expanded clay (grain size 1–5 mm) were used as adsorptive and non-adsorptive filtration media. The filters were operated with an empty bed contact time ranging from 28 to 34 min and a filter velocity of about 3.9–4.8 $m\ h^{-1}$ achieving a net specific throughput of approximately 24,000–27,000 $m^3\ m^{-3}$ bed volumes.

Two pilot scale MBRs consisting of an aerated tank with a submerged membrane (0.04 μm) and a denitrification reactor were operated in parallel fed by preliminary treated wastewater of the WWTP. MBR1 consisted of a subsequent ozone system. A part of the ozonated wastewater was recirculated into the aerated reactor. The ozone system 2 consisted of one bubble column and an equalization tank. Considering a recirculation ratio of 2.0 ± 0.3 (n = 17) the specific ozone consumption in the ozone system 2 was related to the influent flow rate of the MBR-system resulting in a specific ozone consumption of $1.0 \pm 0.2\ g_{O3}\ g_{DOC}^{-1}$ (n = 82). The hydraulic retention time in the bubble column was 27 ± 6 min (n = 92). The sludge retention time was about 57 ± 19 d (n = 32). MBR2 was operated as reference without subsequent ozonation system and recirculation. Further details can be found in the supporting information (SI).

The mechanically treated raw wastewater was not investigated by ecotoxicological tests, as raw wastewater is known to be very toxic for aquatic organisms and it was not the aim to prove again that conventional treatment methods reduce this toxicity (Smital et al., 2011).

2.2. Measurements of physical-chemical parameters in the advanced treatment processes

The following physical-chemical parameters were measured in the effluents of the wastewater treatment reactors, i.e. the nine wastewater streams listed in Table 1: chemical oxygen demand (COD, filtered 0.45 μm), dissolved organic carbon (DOC), nitrite (NO_2-N), nitrate (NO_3-N), ammonium (NH_4-N) and total phosphorus (P_{total}). Details of the applied methods are listed in the SI.

In addition, parameters required by the respective OECD test guidelines were measured directly in the test vessels, namely: temperature and pH in all tests and dissolved oxygen content and total hardness in the tests with *D. magna* and *L. variegatus*. Results are given in the SI.

2.3. On-site biotests

All organisms used in the biotests originated from in-house cultures of ECT Oekotoxikologie GmbH. The tests followed established OECD tests methods and will only briefly be described here. More detailed information can be found in the SI. All tests were carried

Table 1
Overview of the nine investigated wastewater streams together with the origin of the wastewater fed into each process and the codes used for the effluent of the respective treatment step. Except wastewater stream B, all wastewater streams were produced at the pilot plant.

Code	Treatment process	Wastewater feed
B	Conventional activated sludge treatment	Primarily (mechanically) treated
$B+O_3$	Ozonation (ozone system 1)	B
GAC	Granular activated carbon filter, non-aerated	$B+O_3$
$GAC+O_2$	Granular activated carbon filter, aerated	$B+O_3$
BF	Biofilter, non-aerated	$B+O_3$
$BF+O_2$	Biofilter, aerated	$B+O_3$
MBR1	Membrane bioreactor treatment 1	Primarily (mechanically) treated
$MBR1+O_3$	Ozonation (ozone system 2)	MBR1
MBR2	Membrane bioreactor treatment 2	Primarily (mechanically) treated

out on-site at a pilot treatment plant in a flow-through system by constantly transporting undiluted wastewater from the different treatment processes through polytetrafluoroethylene (PTFE) tubes to 10 L stainless steel reservoirs by peristaltic pumps (Otto Huber GmbH, Böttingen, Germany). Each wastewater stream was constantly pumped (Ismatec, Wertheim-Mondfeld, Germany) from its respective reservoir to the test organism's exposure vessels, which were provided with passive overflows. A 5-fold volume exchange rate (calculated renewal of test vessel volume per day) was adjusted for every test vessel in the test with *D. magna,* and a 7-fold exchange rate was applied in the tests with *L. variegatus* and *L. minor*. Test vessels were placed in random order in a water bath that ensured constant temperatures by external heating and cooling units (Julabo, Seelbach, Germany). In each biotest a control flow-through treatment was included with test organism-specific culture medium according to the respective OECD guideline, in the following referred to as medium control.

2.3.1 L. minor

A growth inhibition test with the common duckweed *L. minor* was conducted based on OECD guideline 221. Exposure was run with 4 replicates, each containing four *Lemna* colonies (12 fronds) at test start. Controls were conducted in modified Steinbergís medium (OECD guideline 221). Number and dry weight of fronds per replicate were recorded after 7 days and the response variables growth rate and yield (fronds) were evaluated.

2.3.2 L. variegatus

Two 28-day sediment-water toxicity tests based on OECD guideline 225 were performed with a time lag of five months between the tests. The first test was repeated due to strong effects observed in some treatments as a result of elevated concentrations of ammonium and nitrite in the MBR1 effluent. Both tests were run with six replicates per treatment, inoculating each replicate with 10 synchronized *L. variegatus*. Reconstituted water (OECD 203) served as control medium. The response variables reproduction (total number of living worms) and biomass, measured as ash-free dry weight of worms per replicate vessel were evaluated after the 28 day exposure period in both tests.

2.3.3 D. magna

A reproduction test was conducted with *D. magna* clone M10 based on OECD guideline 211. Exposure was run with 10 daphnids (less than 24 h old at test start) per replicate vessel and four replicates per treatment. Elendt medium M4 was used as control medium. The response variables survival, number of living offspring per surviving female within 21 days, and the intrinsic rate of population growth r (calculated from age-specific lethality and fertility) were evaluated.

2.3.4. D. magna *challenged with parasites*

In parallel to the standard reproduction test and following the same test design, *D. magna* were challenged with spores of the bacterial endoparasite *Pasteuria ramosa* that sterilizes its host in the course of the infection (Ebert et al., 1996). *P. ramosa* coexists with *D. magna* in their natural habitat, but is not expected to occur in wastewater. Infection with *P. ramosa* is macroscopically expressed by sterilization of the host and endospores are microscopically visible in host tissue.

Pasteuria-challenged and placebo-treated daphnids were simultaneously exposed to selected wastewater streams (B, $B+O_3$ and BF) in parallel with *Pasteuria*-challenged daphnids in control medium. Detailed information about the method used to challenge *D. magna* with *P. ramosa* can be found in the SI and in previous publications (Coors and De Meester, 2008, 2011). The infection rate of parasite-challenged *D. magna* was used as endpoint in addition to those of the standard test (see 2.3.3).

2.4. Statistical analysis

Normal distribution of errors and homogenous variances were confirmed visually and by the Leveneís test ($\alpha = 0.05$), respectively, for the response variables *Lemna* growth rate (frond number) and yield (frond number), *Daphnia* offspring, population growth rate, survival and sterilization rate and *Lumbriculus* number of worms and dry weight. Following this the Tukey HSD test was conducted in STATISTICA (version 12, StatSoft Inc.) to test for significant differences between treatments ($\alpha = 0.05$, two-sided) for all response variables mentioned above with the exception of survival of parasite-challenged *Daphnia*, which was tested with the Kruskal-Wallis test due to the lack of homogenous variances.

2.5. Chemical analysis

The elimination of 30 selected micropollutants (including pharmaceuticals, X-ray contrast media, industrial chemicals and three known TPs) in the various treatments steps was investigated in a parallel study over a period of 21 months that also covered the period when the ecotoxicological testing was conducted. For detailed information of the chemical analysis see Knopp et al. (2016).

3. Results and discussion

The validity criteria established by the respective OECD test guidelines were met in each of the biotests. In particular the range of pH, temperature, hardness and oxygen content was within the limits prescribed by the respective test guidelines. This renders it unlikely that effects observed in any of the treatments are caused by these parameters. If not mentioned here, results of measurements of the physical chemical parameters in the effluents of the

treatment reactors (COD, DOC, NO_2-N, NO_3-N, NH_4-N and P_{total}) for all biotests are given in the SI.

3.1. Chemical analysis

Briefly, Knopp et al. (2016) reported that ozonation eliminated a broad range of the investigated micropollutants, with only 11 of the 30 micropollutants being still detectable after the ozonation step. Most of these 11 micropollutants were removed during the subsequent GAC filtration, while less effective elimination was achieved by the biological filtration. Three TPs were identified. Tramadol-N-oxide was formed from the analgesic tramadol during ozonation (average concentration of $0.05 \pm 0.06\ \mu g\ L^{-1}$), and then eliminated by GAC but not by biological filtration. The antiviral drug acyclovir was found to be transformed into carboxy-acyclovir during biological treatment (average concentration of $3.4 \pm 1.4\ \mu g\ L^{-1}$), which was subsequently transformed to COFA (*N*-(4-carbamoyl-2-imino-5-oxo imidazolidin)-formamido-*N*-methoxyacetetic acid, average concentration of $2.6 \pm 1.0\ \mu g\ L^{-1}$) by ozonation. COFA was not removed by GAC or biological filtration.

3.2. Growth inhibition of L. minor

L. minor showed the highest growth rate and yield in the medium control (Fig. 1A and B). There was no indication for any toxification of the wastewater due to the formation of TPs by ozonation since growth in the ozonated wastewater streams (B + O_3, MBR1 + O_3) did not significantly differ from that in the respective non-ozonated streams (B and MBR1). Compared to the medium control, growth was significantly inhibited in the MBR2 and in three of the four wastewater streams that resulted from the complete advanced treatment process of ozonation followed by GAC or biofilters. An unexpected but not significant difference occurred between the non-aerated and the aerated biofilter. Growth in the biologically treated and in the ozonated wastewater stream was intermediate between that observed in the medium control and the advanced treated wastewater, though not significantly different from either of them. Hence, toxicity to *L. minor* increased after a treatment step (filtration, particularly GAC filtration) that is known to remove micropollutants very effectively rather than lead to the formation of TPs. This increase in growth inhibition with intensification of treatment is in contradiction with an expected reduction of growth inhibition due to removal of organic pollutants (Nowotny et al., 2007). The expected removal of organic micropollutants was actually confirmed by the results of the chemical analysis in the parallel study (Knopp et al., 2016) of the here investigated treatment steps. The increased growth inhibition in *L. minor* despite the reduced load of micropollutants may point at nutrient-limitation as reason for the reduced growth due to removal of nutrients by advanced treatment methods and a lack of additional supplementation (e.g. by dilution with usual culture medium). Activated carbon is particularly known to reduce not only the amount of micropollutants in treated wastewater but also nutrient concentration (Bundschuh et al., 2011). Yet, the concentrations of nitrate and phosphorus (see SI, Fig. S1) were highest in the MBR treatments, due to an operation failure in the MBR pilot plants, and much lower in the other treatments (including BF as the treatment with maximum growth rate), which is in contradiction to nutrient limitation as sole explanation for the observed effects. Nitrite and ammonium concentrations were highest in B and BF as well as in B and B + O_3. As no growth inhibition was observed in these treatments, elevated nitrite and ammonium concentrations were not the reason for the observed growth inhibition either. In fact, no significant correlations were found between the biological endpoints (frond number and yield) and any of the measured water parameters (Pearson, $p > 0.05$). Hence, the reason for the observed increasing growth inhibition with intensification of treatment remains unclear, since neither nutrient limitation nor formation of toxic TPs by ozonation can sufficiently explain the observed pattern.

3.3. Reproduction of L. variegatus

Compared to the medium control, the number of *L. variegatus* as a measure of reproduction (Fig. 2A) was significantly reduced in all treatments except the non-aerated GAC, the non-aerated BF and the MBR2 in the first test. An inhibition of 46% compared to the medium control treatment was found for *L. variegatus* exposed to the conventionally treated wastewater (B). Subsequent advanced treatment processes significantly improved reproduction of *L. variegatus* in comparison to B, although rarely up to the level of the medium control. The lowest number of worms was found in the wastewater treated by MBR, particularly MBR1 before and after ozonation. The response variable biomass (Fig. 2B) also showed significant differences between the medium control and the MBR wastewater streams, but no significant differences among the remaining treatments and between them and the medium control. *L. variegatus* showed a significantly reduced reproduction in the conventionally treated wastewater (B) compared to the medium control treatment also in the second test (Fig. 2C). Similarly to the first test, this effect was eliminated after ozonation and in most of the subsequent filtration treatments. In contrast to the first test, the number of worms in the MBR treatments was at the level of the medium control. The only significant differences regarding biomass in the second test occurred between the medium control and B + O_3, MBR1 and MBR1 + O_3 (Fig. 2D).

The improvement of reproduction of *L. variegatus* exposed to the conventionally treated wastewater after ozonation (B + O_3) was simultaneously accompanied by a slight but not significant reduction of biomass in both tests, i.e., *L. variegatus* was able to reproduce at the expense of growth, which may point at impaired nutritional conditions (Leppänen and Kukkonen 1998) or the presence of toxic components in B + O_3 affecting only growth. In several other treatments, significant effects on worm number did not translate into significant effects on biomass.

Concentrations of ammonium and nitrite were strongly elevated in the MBR1 and MBR1 + O_3 wastewater streams compared to all other treatments (all below $1\ mg\ L^{-1}$) during the first exposure period with *L. variegatus* (Fig. 3A and B) most likely due to an insufficient denitrification in the MBR1 reactor as a result of an incomplete run-in time of the MBR reactors. Since in particular ammonium is known for its high toxicity to *L. variegatus* and other aquatic organisms (Egeler et al., 2010; Schubaur-Berigan et al., 1995), these two parameters may explain the observed effects in these treatments. Worm number and biomass both significantly correlated with ammonium and nitrite concentrations (all Spearman $R > -0.88$, all $p \leq 0.001$,), but not with nitrate concentration (Spearman, $p > 0.05$). Yet, ammonium and nitrite were not elevated in the conventionally treated wastewater (below $1\ mg\ L^{-1}$ at the three measurement time points), and therefore cannot sufficiently explain the significantly reduced reproduction in the B treatment compared to the medium control in both tests. However, toxic peak concentrations cannot be excluded securely, due to the limited number of measurements.

During the second test, which was conducted to verify results of the first test, and which was run after a longer establishing time of the MBR reactors, ammonium concentrations in the MBR treatments were below $2.5\ mg\ L^{-1}$ (Fig. 3D) with reproduction being at medium control level, which further supports the role of ammonium in the observed toxicity to *L. variegatus*.

Overall, the two tests with *L. variegatus* provided no evidence that toxic TPs were formed by ozonation at a concentration that would impact the performance of this benthic organism. This

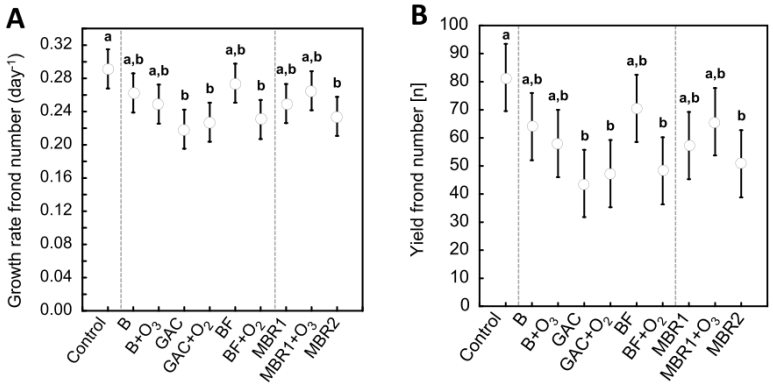

Fig. 1. Growth rate (A) and yield (B) based on frond number of *L. minor* after 7 d exposure to the various wastewater streams (abbreviations as in Table 1) and the medium control. Shown are means with 95% confidence intervals, n = 4 per treatment. Identical letters denote treatments that did not significantly differ from each other (Tukey HSD test, α = 0.05).

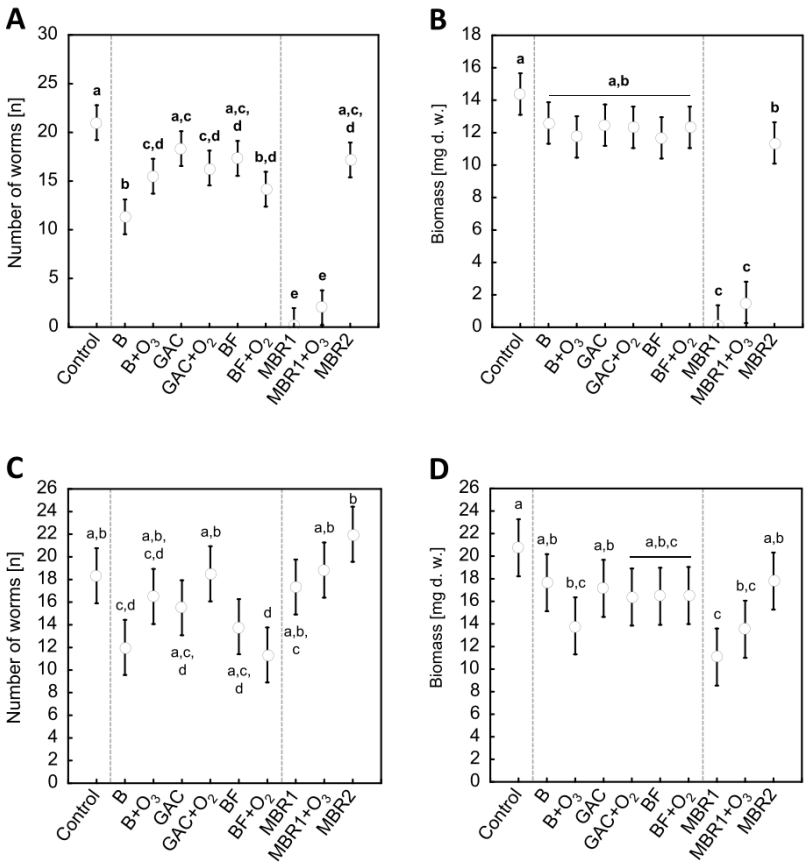

Fig. 2. Reproduction, given as the number of worms (A, C) and biomass given as dry weight (B, D) of *L. variegatus* after the first (A, B) and the second (C, D) test with 28 days exposure period to the various wastewater streams (abbreviations as in Table 1) and the medium control. Shown are means with 95% confidence intervals, n = 6 per treatment. Identical letters denote treatments that did not significantly differ from each other (Tukey HSD test, α = 0.05).

result is in contradiction to earlier studies at different WWTPs (Magdeburg et al., 2012; Stalter et al., 2010a,b). Classic water quality parameters (nitrite and ammonium concentration) were on the other hand identified as most likely reasons for observed effects on *L. variegatus*, which points at the importance of closely monitoring such parameters in whole effluent toxicity tests in order to enable a clear distinction of effects due to toxic nitrogen species resulting from insufficient treatment from effects of potentially toxic micropollutants and their TPs.

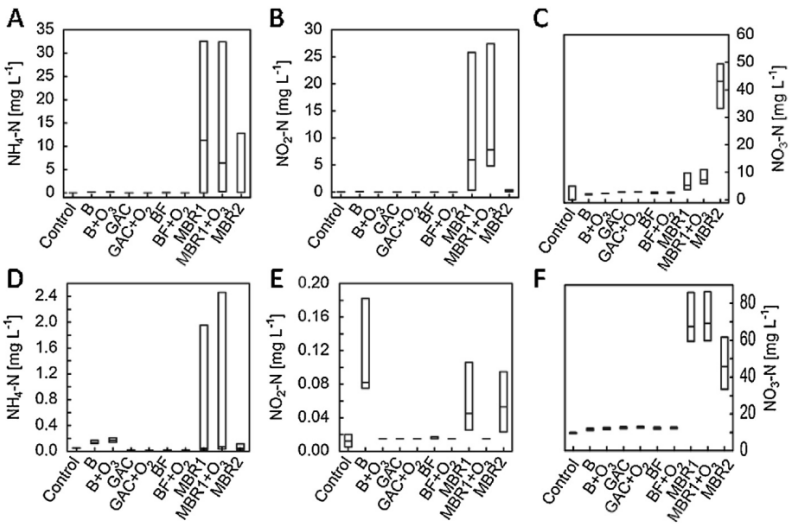

Fig. 3. Concentration of ammonium (NH_4-N), nitrite (NO_2-N) and nitrate (NO_3-N) in the different wastewater streams during the first (A–C) and the second (D-F) test with *L. variegatus*. Medium control values were measured in the test vessels. The boxplots are showing minimum, maximum and median (n = between 3 and 11 measurements per treatment).

3.4. *Survival and reproduction of* D. magna

Mean survival of *D. magna* reached more than 80.0% with no significant differences between the treatments, which demonstrates that the concentration of the complex mixture of micropollutants and TPs was not acutely toxic to *D. magna*. Reproduction did not differ significantly between the medium control and the wastewater streams with exception of the BF + O_2 treatment where the highest offspring number occurred (Fig. 4A). Reproduction was enhanced compared to the medium control, indicating a positive effect of the conventionally treated wastewater. This is most likely due to the presence of bacteria that can be utilized as additional food source by the filter-feeding *D. magna* (Uhlmann 1954; Lampert 1987). Reproduction was reduced in B + O_3 and MBR treatments, although not significantly below medium control level. This could be explained by the reduction of bacterial loads, and hence food supply, by ozonation and the membrane filter. A similar, although slightly less pronounced pattern was found for population growth rate of *D. magna* (Fig. 4B). No significant correlations were found for the biological endpoints and any of the measured physical-chemical parameters (Pearson all $p > 0.1$, Table S5 in SI).

3.5. *Parasite-challenged* D. magna

Parasite-challenge tended to slightly reduce survival of *D. magna* compared to the respective placebo control, except in the case of the ozonation treatment (Fig. 5A). However, there were no significant differences in survival among treatments. Sterilization occurred only in *Pasteuria*-challenged daphnids, and microscopic inspection confirmed that all sterilized daphnids were infected with *P. ramosa*. Sterilization percentages (Fig. 5B) were significantly higher in the *Pasteuria*-challenged medium control than in conventionally or advanced treated wastewater. As a result, the population growth rate was significantly reduced compared to all other treatments in the *Pasteuria*-control (Fig. 5C).

The combined exposure of *D. magna* to treated wastewater and *P. ramosa* did not provide evidence for an immunotoxic effect of the investigated wastewater streams. On the contrary, significantly reduced infection percentages and a tendency for better survival were observed upon wastewater exposure compared to the medium control treatment. This may be explained by *Daphnia* being more resistant to parasite challenge due to improved nutritional conditions in the wastewater treatments, evidenced by higher reproduction in the parasite-free test (see 3.3). Another explanation would be the presence of substances that directly inhibited the parasite *P. ramosa* such as residues of disinfecting ozone or bacteriostatic or bacterio-toxic micropollutants (e.g. antibiotics). *P. ramosa* is susceptible to antibiotics, and *D. magna* has actually been cured from infections by tetracycline treatment (Little and Ebert 2000). Yet, it is currently unknown if *P. ramosa* is indeed susceptible to the typical concentrations of antibiotics in WWTP effluents (low $\mu g\,L^{-1}$ range), which are much lower than the 0.025 $mg\,ml^{-1}$, which were used by Little and Ebert (2000).

4. Conclusions

The present study detected evidence for negative impacts of conventionally treated wastewater on aquatic organisms, namely *L. variegatus*, in accordance with previous work (e.g. Margot et al., 2013; Bundschuh and Schulz 2011a). No impairment by conventionally treated wastewater was observed for *D. magna* and *L. minor*. Performance of aquatic organisms with regard to growth, reproduction and parasite resistance improved with intensification of the wastewater treatment, except in the case of *L. minor*. Improved performance after exposure to ozonated wastewater was also reported in studies with rainbow trout (*Oncorhynchus mykiss*) (Margot et al., 2013) and the amphipod *Gammarus fossarum* (Bundschuh and Schulz, 2011b), indicating that subsequent advanced treatment methods can improve water quality and conditions for respective test organisms. While results of the MBR treatment, especially regarding the first test with *L. variegatus*, are not generally representative for MBRs they demonstrate the harmful effects of elevated ammonium and nitrite concentrations for aquatic organisms as a consequence of an insufficient de-/nitrification (Tchobanoglous et al., 2014). This finding stresses the importance of closely monitoring and reporting classic water quality parameters in studies that aim to investigate the effects of micropollutants and their TPs in wastewater.

In contrast to expectations based on previous studies (Schlüter-Vorberg et al., 2015; da Costa et al., 2014; Magdeburg et al., 2012;

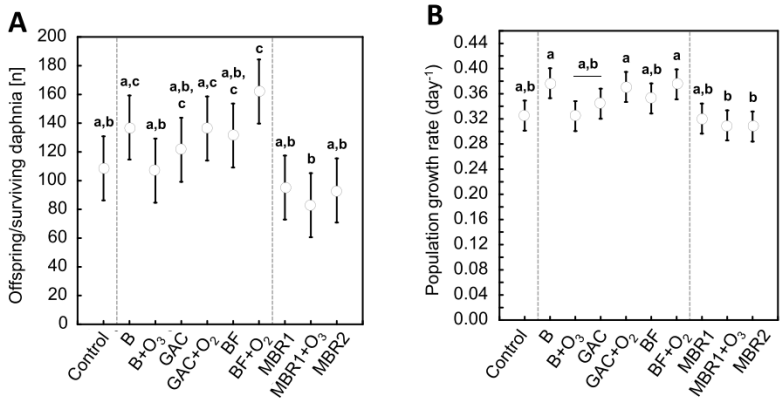

Fig. 4. Reproduction as living offspring per surviving female (A) and intrinsic rate of population increase per day (B) of *D. magna* after a 21 days exposure period to the various wastewater streams (abbreviations as in Table 1) and the medium control. Shown are means with 95% confidence intervals, n = 4 per treatment. Identical letters denote treatments that did not significantly differ from each other (Tukey HSD test, $\alpha = 0.05$).

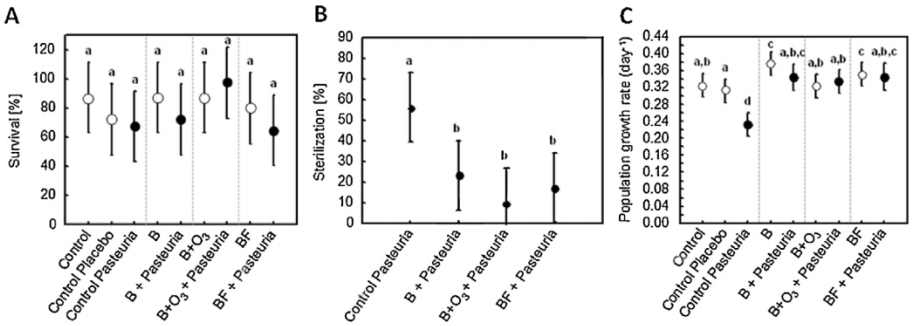

Fig. 5. Survival (A), sterilization percentage (B) and intrinsic growth rate of population increase per day (C) of *D. magna* after the 21 days exposure period to the various wastewater streams (abbreviations as in Table 1) and the medium control with and without challenge by the parasite *Pasteuria ramosa*. Shown are means with 95% confidence intervals, n = 4 per treatment. Identical letters denote treatments that did not significantly differ from each other (A: Kruskal–Wallis test, $\alpha = 0.05$; B, C: Tukey HSD test, $\alpha = 0.05$). White circles indicate *Pasteuria*-free treatments; black circles indicate *Pasteuria*-challenged treatments.

Stalter et al., 2010a, 2010b), none of the three test organisms in the present study provided clear evidence for toxic concentrations of TPs due to ozonation. In a parallel study Knopp et al. (2016) determined three TPs in the here investigated pilot treatment plant, among them COFA (N-(4-carbamoyl-2-imino-5-oxoimidazolidin)formamido-*n*-methoxy-acid), a TP of the antiviral drug acyclovir. COFA was shown to affect growth of the green algae *Raphidocelis subcapitata* (Schlüter-Vorberg et al., 2015), but the measured concentrations in the treated wastewater streams here were at least factor 7 below the EC_{50} of COFA. Hence, this example illustrates that toxic TPs can indeed be formed during advanced wastewater treatment, but not necessarily at concentrations that lead to measurable effects in standard chronic toxicity tests.

In general, a cause-effect relationship between performance of exposed aquatic organisms and potentially toxic TPs in the complex wastewater matrix is difficult to prove, since nutrients as well as classic wastewater parameters (such as ammonium and nitrite) may influence or hinder the observations and the interpretation of results. Additionally, such impacts appear to be species-specific (Schlüter-Vorberg et al., 2015), which points at the importance of a suitable test battery consisting of representatives of different trophic levels and habitats. *L. variegatus* and *L. minor* seemed to be more sensitive than *D. magna* in the present study, keeping in mind the effects were at least partly caused by ammonium and nitrite.

Conflict of interest disclosure

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2017.03.001.

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Supplementary information (SI)

to

Survival, reproduction, growth, and parasite resistance of aquatic organisms exposed *in-situ* to wastewater treated by advanced methods

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1. Characterization of WWTP, pilot treatment plant and wastewater effluents

Pre-selected granular activated carbon (lignite coal, internal surface 1,200 m²/g, grain size 1-4.8 mm, Epibon A, Donau Carbon GmbH, Frankfurt/Main, Germany) and expanded clay (grain size 1-5 mm, AR 1/5-580, ARGEX NV, Zwijndrecht, Belgium) were used as adsorptive and non-adsorptive filtration media.
The specific ozone consumption in the ozone system 1 was 0.9 ± 0.15 g_{O3}/g_{DOC}.
Both MBR consisted of an aerated tank with a submerged membrane (nominal pore size of 0.04 µm, BIO-CEL® BC-10-10-PVC, MICRODYN-NADIR GmbH, Wiesbaden, Germany) and a denitrification reactor.
The specific ozone consumption in the ozone system 2 was 1.06 ± 0.17 g_{O3}/g_{DOC}.

2. Measurements of physical-chemical parameters in the advanced treatment processes

Table S1: Measurements of physical-chemical parameters in the advanced treatment processes

Parameter	Method	Cuvette Test	Spectral photometer	Filter	Disposable syringe
COD	DIN ISO 15705-H45	HACH-LANGE cuvette test LCK 414 (measuring range 5-60 mg O_2/L) HACH-LANGE cuvette test LCK 514 (measuring range 100-2,000 mg O_2/L)	HACH DR 2800 (Hach Lange GmbH, Düsseldorf, Germany)	0,45 µm PES membrane filter (VWR International GmbH, Darmstadt, Germany)	5 mL (Injekt®, B.Braun Melsungen AG, Melsungen, Germany)
DOC	HACH-LANGE purging method (cuvette test)	HACH-Lange cuvette test LCK 385 (measuring range 3-30 mg C/L)			
NH_4-N	Cuvette test according to ISO 7150-1, DIN 38406 E5-1	HACH-LANGE cuvette test LCK 303 (measuring range 2.0-47 mg NH_4-N/L) HACH-LANGE cuvette test LCK 304 (measuring range 0.015-2 mg NH_4-N/L)			
NO_2-N	Cuvette test according to EN ISO 26777, DIN 38405 D10	HACH-Lange cuvette test LCK 341 (measuring range 0.015-0.6 mg NH_4-N/L)			
NO_3-N	Cuvette test according to ISO 7890-1-2-1986, DIN 38405 D9-2	HACH-Lange cuvette test LCK 339 (measuring range 0.23-13.5 mg NO_3-N/L) HACH-Lange cuvette test LCK 340 (measuring range 5-35 mg NO_3-N/L			
PO_4-P/P_{total}	Cuvette test according to ISO 6878-1-1986, DIN 38405 D11-4	HACH-Lange cuvette test LCK 339 (measuring range 0.23-13.5 mg NO_3-N/L) HACH-Lange cuvette test LCK 340 (measuring range 5-35 mg NO_3-N/L			

3. Growth inhibition of Lemna minor

Continuous light during the test with *Lemna minor* was adjusted at 94 µE/m^2 * s. Test temperature was 22.9 to 24.3 °C (mean of 23.5 °C, n = 3 measurements per treatment). The pH ranged from 7.2 to 7.7 in the treatments at test start, which was slightly less acidic than in the control (pH 6.7). At test end, the control had a pH of 7.3 thereby being more in the range of the treatments (pH 7.3 to 8.2). Results of measurements of nitrite (NO_2-N), nitrate (NO_3-N), ammonia (NH_4-N) and total phosphorus (P_{total}) in the effluents of the different treatment reactors during the test with *Lemna minor* are demonstrated in Figure S1. Phosphor was not measured in the vessels of the medium control.

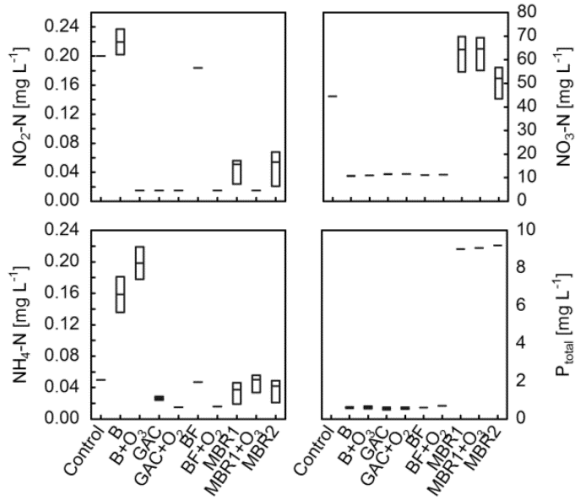

Figure S1: Results of measurements of nitrite (NO_2-N), nitrate (NO_3-N), ammonium (NH_4-N), and total phosphorus (P_{total}) in the effluents of the different treatment reactors during the test with *L. minor*. Control values were measured in the test vessels. Boxplots are showing minimum, maximum and median values.

Results of measurements of the chemical oxygen demand (COD) and the dissolved organic carbon (DOC) are demonstrated in Table S2. No significant correlations were found between the biological endpoints (frond number and yield) and any of the measured water parameters (Pearson, $p > 0.05$).

Table S2: Measurements of the chemical oxygen demand (COD) and the dissolved organic carbon (DOC) in the wastewater streams during the test with *Lemna minor*.

Treatment	COD [mg L^{-1}]	n	DOC [mg L^{-1}]	n
B	34.8 (31.6-45.6)	7	14.2 (12.3-14.5)	7
B+O_3	30.8 (28.1-31.8)	7	13.8 (9.7-14.6)	7
GAC	16.3 (15.3-17.3)	2	7.6 (7.1-8.1)	2
GAC+O_2	14.1 (10.2-18.0)	2	7.6 (7.2-8.0)	2
BF	23.3 (23.1-23.4)	2	10.4 (9.7-11.0)	2
BF+O_2	24.1 (23.2-24.9)	2	10.4 (9.5-11.2)	2
MBR1	14.3 (11.6-17.4)	7	6.7 (6.5-62.2)	7
MBR1+O_3	12.5 (8.1-13.9)	7	6.2 (5.5-6.6)	7
MBR2	22.4 (20.2-24.1)	7	9.2 (8.3-9.9)	7

4. Reproduction of Lumbriculus variegatus

10 synchronized worms were introduced per test vessels (250 mL glass beaker) which were filled with artificial sediment (OECD 225) and overlying test media in a sediment-water ratio of 1 to 4 and a light:dark cycle of 16:8 h with light conditions of about 251 lx (mean) was applied. A mixture of ground nettle powder and alpha-cellulose was added as food to the sediment before start of exposure. Test temperature was 18.0 to 20.7 °C (mean of 19.8 °C, n = 8 measurements per treatment) and 18.9 to 21.0 °C (mean of 19 °C, n = 10 measurements per treatment) during the first and the second test respectively.

The pH was between 7.3 and 8.4 during the first test (mean of 7.8, n = 6 measurements per treatment) and 6.5 and 8.0 during the second test (mean of 7.5, n = 5 measurements per treatment). The dissolved oxygen concentration ranged from 9.1 to 12.2 mg L^{-1} (mean of 10.2 mg L^{-1}, n = 6 measurements per treatment) in the first test and from 6.6 to 15.8 mg L^{-1} (mean of 10.1 mg L^{-1}, n = 5 measurements per treatment) in the second test. Total hardness was between 9.6 and 20.8 °dH (mean of 13.9 °dH, n = 2 measurements per treatment) and between 13.2 and 22.6 °dH (mean of 19.1 °dH, n = 5 measurements per treatment) during the first and the second test respectively. Results of measurements of the chemical oxygen demand (COD) and the dissolved organic carbon (DOC) in the effluents of the different treatment reactors during the tests with *Lumbriculus variegatus* are demonstrated in Table S3 and S4.

Table S3: Results of measurements of the chemical oxygen demand (COD) and the dissolved organic carbon (DOC) in the effluents of the different treatment reactors during the first test with Lumbriculus variegatus, median (min; max).

Treatment	COD [mg L^{-1}]	n	DOC [mg L^{-1}]	n
B	26.4 (18.8; 29.0)	12	11.4 (5.8; 12.6)	12
B+O_3	22.5 (15.60; 25.50)	12	10.9 (7.3; 11.8)	12
GAC	8.9 (7.8; 11.4)	5	5.0 (4.3; 5.2)	5
GAC+O_2	9.3 (7.9; 11.7)	4	5.0 (4.8; 5.3)	4
BF	19.1 (17.8; 20.6)	5	9.1 (8.1; 9.4)	5
BF+O_2	19.1 (17.2; 21.1)	5	9.0 (3.1; 9.2)	5
MBR1	42.7 (2.7; 50.7)	12	11.1 (8.9; 12.5)	12
MBR1+O_3	40.2 (22.2; 53.9)	11	11.7 (8.9; 12.8)	11
MBR2	23.0 (14.1; 32.1)	6	10.1 (3.7; 12.8)	6

Table S4: Results of measurements of the chemical oxygen demand (COD) and the dissolved organic carbon (DOC) in the effluents of the different treatment reactors during the second test with *Lumbriculus variegatus*, median (min; max).

Treatment	COD [mg L^{-1}]	n	DOC [mg L^{-1}]	n
B	30.44 (19.00; 34.00)	19	12.91 (7.79; 18.10)	19
B+O_3	25.90 (17.80; 30.30)	18	12.21 (8.05; 14.20)	18
GAC	14.88 (11.20; 16.70)	8	7.29 (6.11; 8.01)	8
GAC+O_2	14.09 (10.20; 16.80)	8	6.74 (4.83; 7.65)	8
BF	20.08 (16.00; 21.90)	8	9.35 (7.06; 10.10)	8
BF+O_2	20.64 (15.40; 23.40)	8	8.93 (5.39; 9.97)	8
MBR1	16.79 (12.00; 29.20)	17	7.63 (5.85; 12.20)	17
MBR1+O_3	13.58 (8.55; 24.20)	17	7.09 (4.98; 11.10)	17
MBR2	22.12 (17.40; 24.20)	11	9.79 (4.70; 18.70)	10

5. Survival and reproduction of *Daphnia magna*

Daphnia magna clone M10 was used in this reproduction test. A light:dark cycle of 16:8 h was adjusted by 288 lx (mean). Test temperature was 19.9 to 20.9°C (mean of 20.4°C, n = 4 measurements per treatment). Elendt medium M4 (Elendt 1990) was used in the negative control treatments. Daphnids were fed green algae suspension (*Desmodesmus subspicatus*) daily with 0.15 mg C/daphnia from day 0 until day 6 and 0.25 mg C/daphnia from day 7 onwards. The pH ranged from 6.3 to 7.9 (mean of 7.2, n = 4 measurements per treatment), the dissolved oxygen concentration in the test vessels was between 3.7 and 20.6 mg L^{-1} with lowest values in the MBR1 and highest values in the B+O_3 replicates (mean of 10.6 mg

L^{-1}, n = 4 measurements per treatment) and total hardness was between 14.2 and 19.8 °dH (mean of 16.7 °dH, n = 4 measurements per treatment). Survival of daphnids was checked and produced offspring were counted and removed from test vessels daily. The response variables survival, number of living offspring per surviving female within 21 days, and the intrinsic rate of population growth r (calculated from age-specific lethality and fertility according to Lotka 1913) were calculated.
Results of measurements of the chemical oxygen demand (COD), the dissolved organic carbon (DOC), ammonia (NH_4-N), nitrite (NO_2-N), nitrate (NO_3-N), and phosphor (P_{total}) in the effluents of the different treatment reactors during the test with *Daphnia magna* are demonstrated in Table S5.

Table S5: Chemical oxygen demand (COD), the dissolved organic carbon (DOC), ammonia (NH_4-N), nitrite (NO_2-N), nitrate (NO_3-N), and phosphor (P_{total}) in the effluents of the different treatment reactors during the tests with *Daphnia magna*, median (min; max).

treatment	COD [mg L^{-1}]	n	DOC [mg L^{-1}]	n	NH_4-N [mg L^{-1}]	n	NO_2-N [mg L^{-1}]	n	NO_3-N [mg L^{-1}]	n	P_{total} [mg L^{-1}]	n
B	27.76 (19.70; 32.60)	16	11.14 (7.80; 12.40)	16	0.26 (0.12; 0.49)	3	0.34 (0.24; 0.54)	3	1.99 (1.63; 2.70)	3	0.52 (0.43; 0.64)	3
B+O_3	23.72 (16.40; 29.00)	16	11.20 (8.05; 16.70)	16	0.29 (0.14; 0.52)	3	0.02 (0.02; 0.02)	3	2.55 (1.99; 3.67)	3	0.51 (0.44; 0.62)	3
GAC	12.04 (6.30; 17.10)	6	6.07 (4.36; 7.42)	6	0.06 (0.03; 0.12)	3	0.10 (0.02; 0.25)	3	2.82 (2.15; 3.88)	3	0.42 (0.26; 0.56)	3
GAC+O_2	15.14 (13.00; 18.00)	5	7.13 (6.51; 8.29)	5	0.02 (0.02; 0.02)	3	0.02 (0.02; 0.02)	3	3.05 (2.38; 4.22)	3	0.50 (0.48; 0.51)	3
BF	19.70 (17.50; 22.60)	6	9.20 (8.60; 9.80)	6	0.22 (0.09; 0.36)	3	0.22 (0.16; 0.32)	3	2.41 (1.91; 3.37)	3	0.48 (0.43; 0.58)	3
BF+O_2	20.35 (18.60; 21.10)	6	9.19 (8.40; 10.50)	6	0.09 (0.02; 0.23)	3	0.02 (0.02; 0.02)	3	2.80 (2.14; 4.06)	3	0.53 (0.46; 0.58)	3
MBR1	16.16 (10.20; 21.90)	14	7.23 (5.85; 9.25)	15	1.61 (0.02; 10.80)	11	0.19 (0.01; 1.00)	12	49.02 (27.20; 71.20)	12	6.27 (5.90; 6.64)	2
MBR1+O_3	12.92 (8.66; 17.00)	15	6.65 (4.78; 8.45)	15	1.19 (0.02; 7.39)	11	0.02 (0.02; 0.06)	12	50.19 (29.00; 70.60)	12	6.23 (5.95; 6.51)	2
MBR2	21.41 (11.10; 29.30)	16	9.15 (7.63; 12.70)	15	5.11 (0.02; 26.70)	13	0.19 (0.02; 0.60)	12	45.65 (7.56; 68.00)	12	7.04 (6.98; 7.10)	2

6. Parasite-challenged *Daphnia magna*

On day 0 *Pasteuria*-treatments received 1 mL of a solution containing $2 \cdot 10^5$ endospores of *P. ramosa* added to 19 mL exposure medium. A placebo-control, consisting of M4 medium and a placebo-solution (containing an amount of ground daphnia tissue equal to the *Pasteuria*-solution), and a *Pasteuria*-control, containing M4 and *Pasteuria ramosa* were investigated besides the negative control and the wastewater treatments. Exposure from day 0 to 3 was conducted statically in 20 mL exposure media. On day 4 all daphnids were transferred to 1 L-glass beakers and from then on exposure was carried out in the flow through system without adding of new *Pasteuria ramosa* spores. Daphnids were fed green algae suspension (*Desmodesmus subspicatus*) with 0.1 mg C/daphnia once on day 0. They received 0.15 mg C/daphnia from day 4 until day 6, and 0.25 mg C/daphnia from day 7 onwards. Test temperature in the test vessels ranged from 18.4 to 20.8°C (mean of 20.1°C, n = 4 measurements per treatment). The pH was between 6.3 and 7.9 (n = 4 measurements per treatment). The dissolved oxygen concentration in the test vessels was 5.6 to 18.7 mg L-1 (mean of 10.2 mg L-1, n = 4 measurements per treatment) throughout the test period and total hardness ranged between 14.2 and 17.4 °dH (mean of 15.6 °dH, n = 4 measurements per treatment).

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I.III **Impact of an immunosuppressant on the interaction of a bacterial parasite and its invertebrate host**

Schlüter-Vorberg, L., Coors, A., submitted 08/2018.

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LS: 100%; Collecting of data and preparation of figures and tables regarding ecotoxicity experiments

Analysis of data:

LS: 65%; Statistical analysis and interpretation of data regarding ecotoxicity experiments

AC: 35%; Support with statistic-expertise and interpretation of data

Drafting of the manuscript:

LS: 70%; Writing of the manuscript

AC: 30%; Correction and revisions

Impact of an immunosuppressant on the interaction of a bacterial parasite and its invertebrate host

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Abstract

The interaction of pollutants and pathogens may result in altered and often enhanced effects of the chemical, the biotic stressor or both. These interaction effects cannot be reliably predicted from the toxicity of the chemical or the virulence of the pathogen alone. While standardized detection methods for immunotoxic effects of chemicals exist with regard to human health, employing *host-resistance assays* with vertebrates, such standardized test systems are completely lacking for invertebrate species. In the present study, we investigated the impact of the immunosuppressive pharmaceutical cyclosporine A (CsA) on the invertebrate host-pathogen system *Daphnia magna – Pasteuria ramosa*. CsA is a calcineurin-inhibitor and also known to have antibiotic as well as antifungal properties. Juvenile *D. magna* were exposed to CsA for 21 days with additional pathogen challenge during the first 72 h. Long-term survival of the host *D. magna* was synergistically impacted by co-exposure to the chemical and the pathogen, expressed e.g. in significantly enhanced hazard ratios. Additionally, enhanced virulence of the pathogen upon chemical co-exposure was expressed in synergistically increased infection rates and an increased speed of *Pasteuria*-induced host sterilization. In contrast, effects on reproduction were additive in *Pasteuria*-challenged, but finally non-infected *D. magna.* The integrative endpoint population growth rate reflected synergism, shown by the significant interaction term of both stressors (CsA**Pasteuria*).
The present study provides clear evidence that a pharmaceutical intended to suppress the human immune system, can exhibit immunotoxic effects in an aquatic invertebrate organism. While the exact mechanisms leading to synergism are not fully known, CsA may affect defense responses of *D. magna* against *P. ramosa,* such as phagocytosis or the Toll-like receptor.
While it remains open whether CsA concentrations in the environment are high enough to trigger adverse effects in environmental organisms, our findings highlight the need to consider immunotoxicity in an environmental risk assessment, and to develop suitable standardized methods for this purpose.

Keywords

Daphnia magna; *Pasteuria ramosa;* cyclosporine A; immunotoxicity; synergism

1 Introduction

Since every organism in almost every ecosystem is co-existing with a multitude of pathogens and parasites, an intact immune system is essential for the defense against them to ensure survival of individuals and preservation of populations (Blaustein et al., 2012). Anthropogenic pollutants, that are to date ubiquitos in nearly every ecosystem (Margot et al., 2013), may impact this co-existince by interfering with the functioning of the host's immune system leading e.g. to an increased susceptibilty to pathogens (Galloway and Depledge, 2001; Ebert et al., 2016).

Besides well-known examples for immunotoxic substances among metals, polycyclic aromatic hydrocarbons and pesticides (Ross et al., 1996; Galloway & Handy, 2003), there are pharmaceuticals that intentionally affect the immune system of the human patient, so-called immunosuppressants. They are used, for example, for the treatment of autoimmune diseases or to avoid allograft rejection after transplants. If such drugs enter the environment in their active form, e.g. due to incomplete degradation either in the human body or during wastewater treatment, they could potentially disrupt the immune system of environmental organisms. Currently established regulatory requirements regarding the environmental risk assessment of pharmaceuticals (and other substances) do not include an assessment of immunotoxicity for non-target organisms.

So far, effects of immunosuppressive substances have mainly been tested on vertebrates, in particular mammals, in order to assess possible risks to humans (Marcogliese & Pietrock, 2011). Measurements of various general and specialized immunological functional parameters, such as number and activity of lymphocytes, serve as indicators of immunotoxic effects. However, the immune system is highly redundant, which implies that a change in an immunological parameter does not necessarily translate to an impaired overall immune response. Therefore, so-called *host resistance assays*, where a test organism is simultaneously challenged with a pathogen and the potentially immunotoxic substance, are considered the "gold standard" for the definitive identification of a substance as immunotoxic (Descotes, 2006). The increased virulence of the applied pathogen indicates an immunotoxic effect of the substance due to a weakend host immune system (Descotes, 2005). Virulence is understood as the capacity of a pathogen to cause disease in a host, dependent on the host susceptibility (Casadevall & Pirofski, 2001). As there is no absolute measure, virulence is a relative term and defined by the severity of the caused effect in the host (Pirofski & Casadevall, 2012). Increased pathogen virulence is expressed as increased infection rate in the host population, but may also be manifest in the impairment of other endpoints, such as host survival (Descotes, 2006; Nathanson, 2007).

Since invertebrates account for approximately 95% of all species and play important roles in ecosystems, the potential consequences of immunotoxic environmental pollutants on invertebrate populations and their host-pathogen interactions should be considered (Galloway and Depledge, 2001). Standardized *host resistance assays* with invertebrates are currently not available, which is why investigations of immunotoxicity in invertebrates still relies mostly on the assessment of functional parameters, such as measurements of lysozyme activity, phagocytosis, total and differential hemocyte count or oxyradical generation (Galloway and Depledge, 2001). Despite the lack of standardized assays, several publications describe the

application of invertebrate *host resistance assays* to examine the combined effects of pathogens and pollutants (e.g. Coors & De Meester, 2011; De Coninck et al., 2013; Retschnig et al., 2014). In the present study, we investigated the impact of an immunosuppressant on the invertebrate host-parasite system *Daphnia magna – Pasteuria ramosa*. This research aims to improve knowledge on immunotoxicity in invertebrates in general, and specifically to further explore the potential of the chosen system as invertebrate *host resistance assay*.

The immunosuppressant substance cyclosporine A (CsA) belongs to the group of calcineurin inhibitors. It is mainly applied to suppress the innate immune response of allograft rejection after transplantations in humans and has been used for more than three decades (Fiolka, 2008). Unlike vertebrates, invertebrate organisms possess no adaptive but only an innate immune system similar to the innate immune responses of vertebrates. This includes immunocytes, which eliminate foreign cells from the body by phagocytosis, encapsulation or granuloma formation as well as humoral mechanisms in the hemolymph. These consist for example of antimicrobial polypeptides, agglutinins and opsins, which play a role in the proliferation of immunocytes (Galloway & Depledge, 2001). Little is known about the exact molecular defense mechanisms of *D. magna* in response to *P. ramosa* infection (Ebert et al., 2016). Yet, the phenoloxidase (PO) activity is known to be involved in the defense system against invading pathogens such as *P. ramosa* (Pauwels et al., 2010) and an increasing number of phagocytic cells in the host was shown to be positively correlated with applied spore doses of *P. ramosa* (Auld et al., 2012). CsA was chosen as chemical stressor, due to its known immunosuppressive effects on innate immune functions in vertebrates and invertebrate species such as the inhibition of phagocitic, cytotoxic and antibacterial peptides activity (Vecchiarelli et al., 1989; Vilcinskas et al., 1999; Fiolka, 2008).

In the present study, the hypothesis was tested that CsA-induced immunosuppression leads to an increased virulence of the obligate *Daphnia*-parasite *P. ramosa*, expressed e.g. in synergistically reduced survival, reduced fecundity or increased sterilization rates in *D. magna*.

2 Material and Methods

The potential immunotoxicity of CsA was investigated in two independent *host resistance assays* with the invertebrate host *D. magna* and the bacterial endoparasite *P. ramosa*.

2.1 Host parasite system

The *D. magna* Straus (Crustacea: Cladocera) clone M10 (Cousyn et al., 2001) used in the present study is known to be moderately susceptible to the gram-positive bacterium *P. ramosa* (Coors & de Meester, 2008, 2011). *P. ramosa* sterilizes its host in the progress of the infection. Transmission of endospores occurs strictly horizontal with infective spores being released only from decaying hosts. Endospores of *P. ramosa* are microscopically visible in ground host tissue and the advanced infection is also macroscopically visible by sterilization of the host (Ebert et al., 1996). To obtain *P. ramosa* spores, neonates of *D. magna* clone M10 were exposed to sediments of a pond that has a proven history of *P. ramosa* presence (Jansen et al., 2010). Infected daphnids were ground up after 28 days (time needed for full maturation of parasite spores) and the resulting solution was filtered (60 mm nylon filters, Millipore) and diluted to obtain a standardized spore solution containing $2*10^6$ endospores/ml. A placebo-solution was

prepared in the same way with ground non-infected stock-culture daphnids diluted in deoinized water, containing an equal amount of daphnia tissue as the spore solution. Placebo-solution was applied in all treatments without *Pasteuria*-challenge, and was used as dilution medium of the *Pasteuria* stock-solution in the first experiment. Due to enhanced mortality observed in the placebo-control in the first experiment, the spore-solution was diluted with Elendt medium M4 in the second experiment. *Pasteuria*- and placebo-solutions were stored frozen at -80°C prior to use.

In a previous test with *D. magna* challenged by a range of ten different *P. ramosa* spore doses, the spore quantity was determined at which a 50% infection rate in the challenged *Daphnia* population was observed (ID_{50} = $1*10^5$ spores/ml). The spore dose in the experiments of the present study was accordingly selected in order to achieve an infection rate of 50% that would allow detecting enhanced as well as decreased virulence due to CsA.

2.2 Test substance

Cyclosporine A (CsA, $C_{62}H_{111}N_{11}O_{12}$, CAS 59865-13-3, purity ≥ 98.5%) was obtained from Sigma-Aldrich, Germany. The substance has a $\log K_{ow}$ of 3.64. It has a reported maximum water solubility of 120 mg/l (Ismailos et al., 1991) and is dominantly present (99%) as neutral species despite the numerous polar functional groups. CsA inhibits calcineurin, production of lymphokines and the release of interleukin and is also known to have antibiotic as well as antifungal properties (Kümmerer, 2004).) The EC_{50} for acute immobilisation of *D. magna* is reported with 20 mg/l (Kümmerer, 2004) and 29.6 mg/l (Campos et al., 2014) based on nominal concentrations.

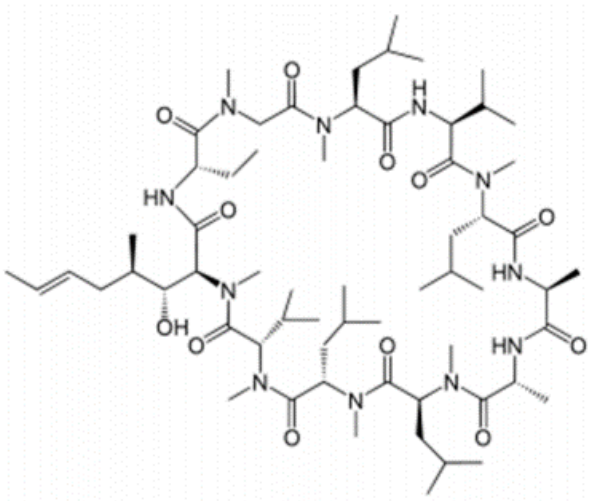

Figure 1: Molecular structure of cyclosporine A.

Due to the large size of the molecule and the numerous polar functional groups, the development of an analytical method is very complicated. A recently developed ultra-performance liquid chromatography using tandem mass spectrometry (UPLC-TMS) method for monitoring CsA levels in blood samples of patients (Lee, 2013) has a limit of quantification (2.5 ng/ml blood), which is within the range of the here tested nominal CsA concentrations (0.001 – 1.0 mg/l). Yet, adaptation and application of this UPLC-TMS method

was beyond the scope of this project. An attempt was made to verify exposure concentrations via measurement of TOC (total organic carbon) in test solutions without *Daphnia* and green algae (provided as food source). However, reliable TOC contents could not be determined due to the small TOC increase due to the low CsA concentrations compared to TOC present in the medium and added by the solvent. Hence, here determined effect concentrations relate to nominal CsA concentrations, and it needs to be kept in mind that actual CsA concentrations in the exposure solutions might have been lower due to sorption or degradation of the substance.

2.3 Experimental setup

Two experiments were performed, each with five concentrations of CsA separated by a spacing factor of 3.2. Concentrations ranged from 0.01 to 1.0 mg/l and from 0.001 to 0.1 mg/l in the first and the second test, respectively. Hence, three identical CsA concentrations (0.01, 0.032 and 0.1 mg/l) were tested in both experiments. Elendt medium M4 (OECD 2012) was used for the controls and as dilution medium for test solutions.
Dilution series of CsA were prepared in organic solvents (ethanol in experiment 1 and acetone in experiment 2). The solvent was changed due to concern on possible effects of ethanol on the host-parasite interaction. The solvent solutions with CsA were stored at - 20°C and test solutions were freshly prepared at each water exchange by adding 250 µl (first experiment) or 500 µl (second experiment) of the respective solvent solution to 1 L of medium.
A blank control (containing M4 only), a placebo-control (containing M4, solvent and placebo solution) as well as a *Pasteuria*-control, (containing M4, solvent and spore solution) were investigated in both experiments along with the CsA treatments. There were three replicate vessels per treatment and each control in the first experiment and four replicate vessels per treatment and controls in the second experiment. Each replicate vessel initially contained 10 *D. magna,* less than 24 h old at test start.
D. magna was challenged with *P. ramosa* spores for the first 72 h only, at a concentration of $1*10^5$ endospores in 20 mL of test solution (with or without CsA). All placebo treatments received placebo solution instead of spore solution. After the first 72 h, daphnids were transferred to 1000 ml test beakers (containing 800 ml test solution), and subsequently exposed semi-statically until day 21, with water renewal three times per week. Daphnids were fed with the unicellular green algae *Desmodesmus subspicatus* at every water exchange. The food level was equal to 0.01 mg C/daphnia given once on day 0, and increased thereafter to 0.1 mg C/daphnia/day from day 4 to 6, and 0.2 mg C/daphnia/day from day 7 onwards. In concordance with previous studies (e.g. Coors et al., 2008; Jansen et al., 2011), low volumes of exposure medium, in combination with relatively high loads of endospores of *P. ramosa* and restricted food level were choosen to create strong infection conditions.

Survival and sterilization status of test animals were recorded daily. Adult *D. magna* were counted as sterilized upon macroscopic inspection if no eggs were visible in the brood pouch and the ovaries. Infection was confirmed microscopically by examination for endospores in the ground tissue at 40-fold magnification at test end (or upon death during the test). Infections occurred solely in the treatments with *Pasteuria* challenge. Earliest microscopically visible infection of *D. magna* in both experiments occurred on day 4 and was determined in ground

tissue of deceased daphnids with visible spores of *P. ramosa*. From day 7 onwards infection was also macroscopically visible in living daphnids, due to brownish colored tissue and the visible sterilization.

Offspring were counted and removed from test vessels daily. To calculate offspring per surviving female in each replicate vessel, the daily sum of living neonates was divided by the number of non-sterilized adult *D. magna* alive at that day. Hence, assessment of reproduction excludes the sterilizing effects of *P. ramosa* infection.

The intrinsic rate of natural increase (population growth rate *r*) was calculated according to (Lotka, 1913). This variable integrates mortality, reproduction and sterilization since the calculation of the age-specific fecundity included all living *D. magna*, regardless of sterilization.

D. magna of the blank control treatments showed a survival of 90% in the first and 87.5% in the second experiment. Survival of placebo- and *Pasteuria*-controls did not significantly differ from that of the respective blank controls in both tests (Dunnett's test, $p<0.05$). Mean offspring number per surviving *D. magna* in the placebo-controls were 108 in the first and 119 in the second experiment. The experiments were conducted at 19.3-21.6°C (mean: 20.4 °C, first experiment), and 19.4- 20.5 °C (mean: 19.9 °C, second experiment), under diffuse light (mean 813 and 735 lx, light/dark cycle of 16/8h). The physicochemical parameters were measured once per week in at least one replicate vessel of fresh and aged test medium. Oxygen was between 6.3–9.1mg/l and 7.9–9.4mg/l; pH was between 7.6–8.3 and 7.1–8.3 in the first and the second experiment respectively. Total hardness was above 240mg/l (as $CaCO_3$) in both tests. Hence, regarding environmental conditions and performance of the controls, both experiments are deemed valid according to OCED guideline 211 (OECD, 2012).

2.4 Statistical analysis

The following data were statistically evaluated: (i) survival curves and resulting hazard ratios, (ii) survival rate at day 10 and at day 21, (iii) *Pasteuria*-induced sterilization rate at day 10 and at day 21, (iv) number of living offspring per surviving *D. magna* until day 21 and (v) population growth rate *r*. Survival and sterilization rate were investigated at day 10 (end of juvenile phase) additionally to day 21 (test end) as a measure for the speed of the infection process. Sterilization or mortality before maturation and first reproduction (occuring around day 10) has strong effects on population growth and can thereby serve as an indicator for virulence.

Survival curves were analysed using the Cox proportional hazard (CPH) model in R (version 3.4.3) with the survival package (version 2.42-6) (Therneau & Lumley, 2015). Censored data (survival until day 21) were handled by the Efron method of the survival package. Confidence intervals (95%) represent robust jackknife estimates. The interaction effect of *Pasteuria* challenge and CsA exposure was tested by using the binary covariates (absence/presence) as linear predictors in a CPH analysis followed by a Wald test. In addition, hazard ratios for each CsA treatment with and without addition of *P. ramosa* were determined in relation to the baseline of the respective placebo control. Hazard ratios >1 indicate an increased risk of death compared to the respective placebo control.

All other data listed above were analyzed by one-way or two-way ANOVA followed by Dunnett's post hoc test in STATISTICA (version 13.3, TIBCO Software Inc. (2017)) to determine Lowest Observed Effect Concentrations (LOECs) and resulting No Observed Effect Concentrations (NOECs). Normal distribution of errors and homogenous variances were confirmed visually and by the combined Cochran, Hartley, Bartlett's test ($\alpha = 0.01$).
Due to partly significant differences of the controls between the two experiments, results for all response variables were related to their respective treatment control (% of placebo- or *Pasteuria*-control), pooled for the two experiments, and fitted by a 2-parameter log logistic model to estimate 10 and 50% effect concentrations (EC_{10} and EC_{50}, respectively) in R using the drc package (Ritz et al., 2015). Figures of fitted dose-response curves are provided in the supplementary information (SI).

3 Results

3.1.1 Survival

The survival of *D. magna* was strongly impacted in both experiments (Fig. 2). The interaction (CsA**Pasteuria*) was marginally not significant in the first experiment (p=0.089, Wald test), but significant in the second experiment with lower CsA concentrations (p=0.007). The single factors (CsA and *Pasteuria*) were significantly affecting survival in both experiments (all $p<0.001$). Hazard ratios for the individual treatments (Tab. 1) indicated no significant impact of *Pasteuria* challenge on survival in the absence of CsA. Yet, survival probability was significantly reduced in combination with *Pasteuria* challenge down to CsA concentrations of 0.001 mg/l, while in the absence of the parasite CsA significantly impacted survival only down to the 10-fold higher concentration of 0.01 mg/l. In general, survival in the control-treatments was more affected in the first experiment, with a mean long-term survival of only 77% and 70% in the placebo- and in the *Pasteuria*-control respectively. In contrast, survival in the second experiment was 95% and 90% (placebo- and *Pasteuria*-control, respectively) until day 21 (Fig. 3). Consistent with the CPH survival analysis, the estimated EC_{50_d21} values indicate an about 12-fold greater toxicity of CsA in the presence of *Pasteuria* challenge compared to placebo treatment (Tab. 3). Survival until day 10 was significantly reduced compared to the control at the highest CsA concentration only in the presence of *Pasteuria* challenge (Fig. 3), which additionally indicates an increase in virulence in terms of speed of the infection impact.

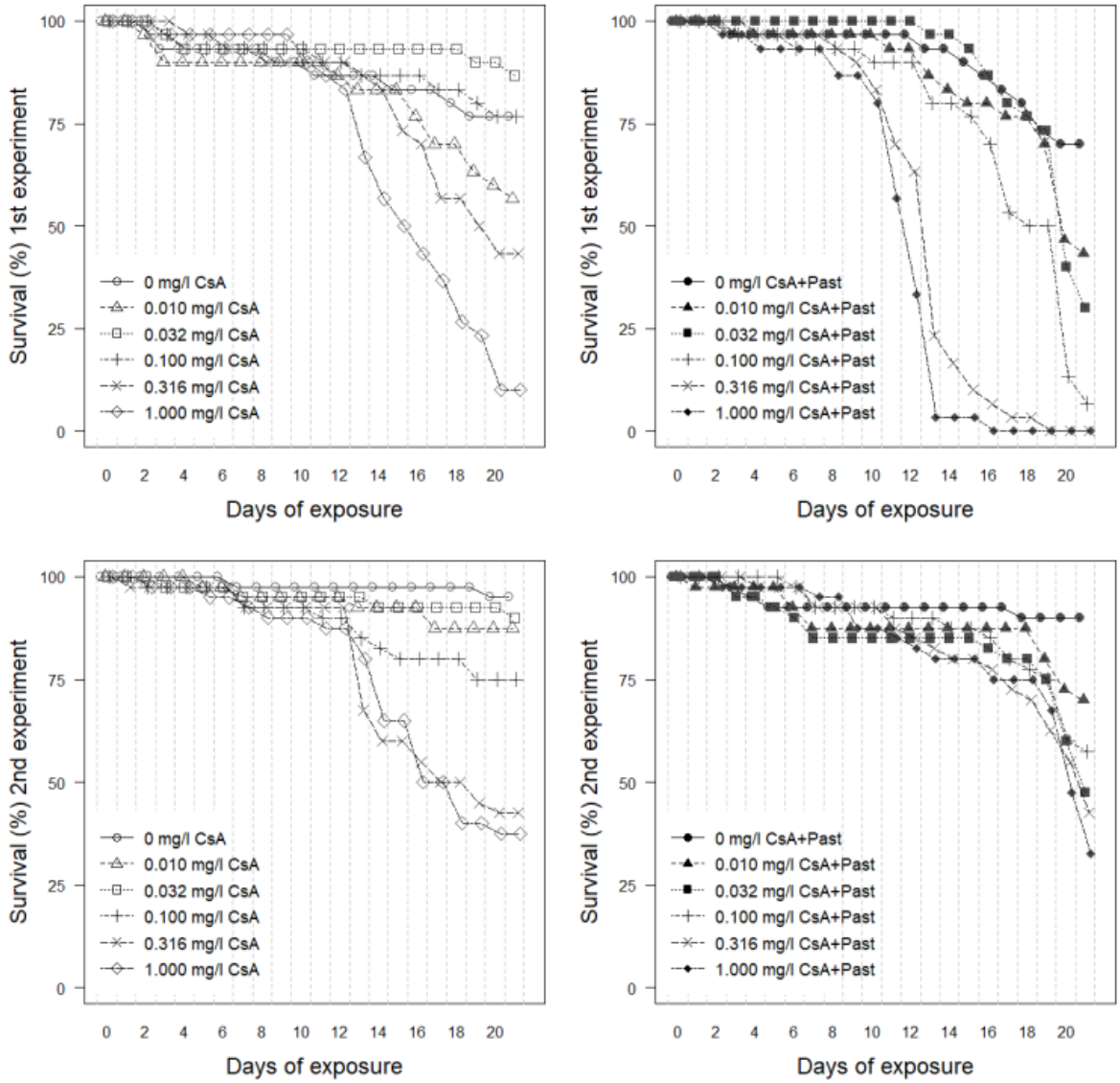

Figure 2: Survival of *D. magna* over 21 days in the two experiments (top, bottom) with placebo- (white symbols) and *Pasteuria*-challenge (black symbols).

Table 1: Hazard ratios determined in the two experiments for the individual treatment combinations of CsA exposure and *Pasteuria* challenge. Survival of the respective placebo controls was taken as baseline. Significance of hazard ratio from CPH model: *** p≤0.001; ** p≤0.01; * p≤0.05.

	Hazard ratios (95% confidence interval)			
	1st experiment		2nd experiment	
CsA (mg/l)	Placebo	*P. ramosa* challenge	Placebo	*P. ramosa* challenge
0.0	1	1.3 (0.5 - 3.6)	1	2.1 (0.4 - 11.6)
0.001	n.t.	n.t.	2.6 (0.5 - 13.6)	7.2 (1.6 – 32.0) **
0.0032	n.t.	n.t.	2.1 (0.4 - 11.3)	14.0 (3.3 - 59.6) ***
0.01	2.1 (0.8-5.1)	3.1 (1.3 - 7.4) *	5.8 (1.3 - 26.4) *	11.0 (2.5 - 47.2) **
0.032	0.5 (0.2 - 1.8)	3.9 (1.6 - 9.2) **	16.6 (4.0 - 70.5) ***	16.3 (3.8 - 69.1) ***
0.1	1.0 (0.3 - 2.8)	8.2 (3.6 - 19.1) ***	17.9 (4.2 - 75.7) ***	19.7 (4.7 - 82.9) ***
0.32	3.0 (1.2 - 7.1) *	44.2 (18.1 - 108.2) ***	n.t.	n.t.
1.0	6.7 (2.9 - 15.6) ***	79.2 (31.4 - 199.8) ***	n.t.	n.t.

n.t.: not tested

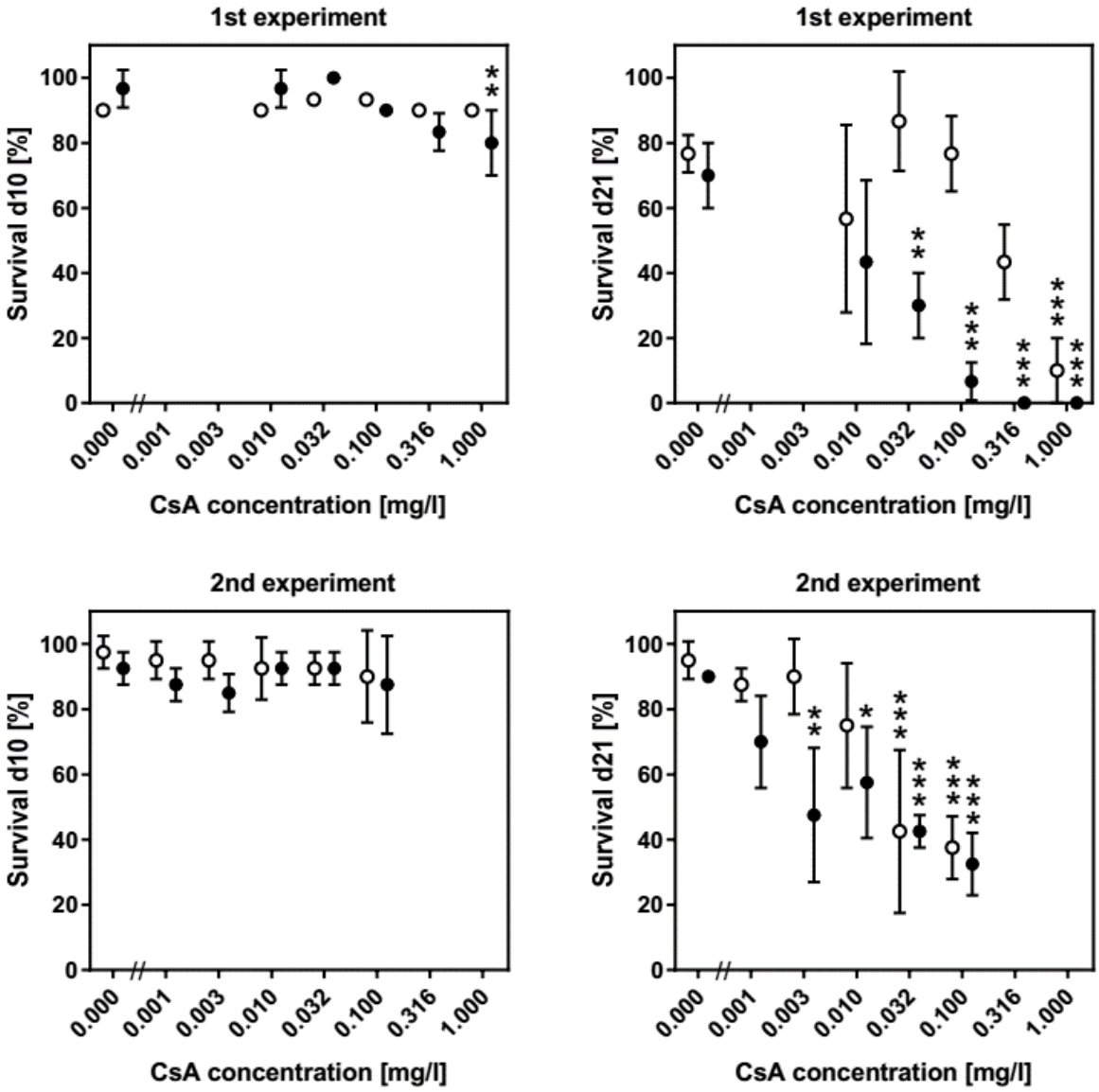

Figure 3: Survival of *D. magna* until day 10 (left) and until day 21 (right) in dependence of CsA alone (Placebo, white symbols) or in combination with *P. ramosa* challenge (black symbols). Shown are means per treatment with their standard deviations in the first (top) and the second experiment (bottom). Significant differences between each treatment and its respective CsA-free control (placebo or *Pasteuria*) and between controls: * $p<0.05$; ** $p<0.01$; *** $p<0.001$ (Dunnett's test).

3.1.2 Sterilization

Microscopic inspections confirmed that all and only infected *D. magna* were sterilized at day 21. The infection rate (which was therefore equivalent to the sterilization rate) was 33% and 45% in the control treatments at test end (first and second experiment respectively) and thus slightly lower than the intended 50%.

The *Pasteuria*-induced sterilization of *D. magna* was strongly impacted in the presence of CsA (Fig. 4). Although lower CsA concentrations were applied, the sterilization rate of *D. magna* was more affected in the second experiment, with significant effects down to the lowest tested CsA concentration (i.e. LOEC ≤0.001 mg/l), while the LOEC determined in the first experiment was 0.1 mg/l (Fig. 4). The sterilization EC_{50} estimate was lower than the survival EC_{50} in presence of the parasite, indicating a stronger effect of CsA on the infection process than on host survival (Tab. 3). A substantial number of daphnids was sterilized prior to or immediately after release of their first brood, with 60% of introduced daphnids sterilized already at day 10 when being simultaneously exposed to 1.0 mg/l CsA (Figure 4). According to results of the one-way ANOVA (Tab. 2), CsA was significantly affecting sterilization rate at day 21 in both experiments ($p<0.01$), as well as at day 10 in the first experiment ($p<0.05$), indicating an increase in speed of sterilization as a measure of enhanced virulence of *P. ramosa*.

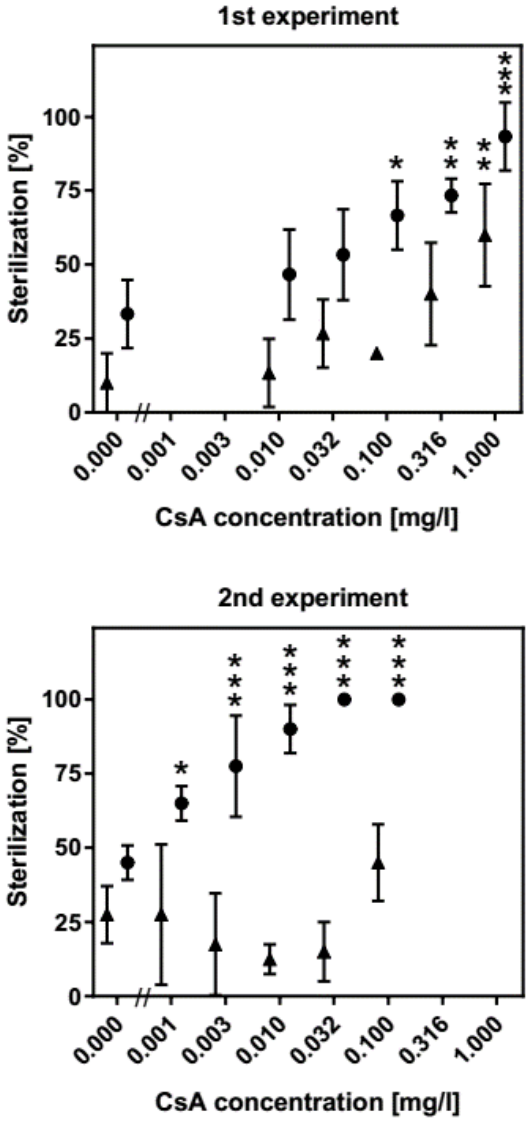

Figure 4: *Pasteuria*-induced sterilization rate in % of introduced *D. magna* at day 10 (triangles) and at day 21 (circles). Shown are means per treatment with their standard deviations in the first (top) and the second experiment (bottom). Significant differences between each treatment and its respective control: * $p<0.05$; ** $p<0.01$; *** $p<0.001$ (Dunnett's test).

3.1.3 Reproduction

The number of offspring per surviving *D. magna* was slightly (but not significantly) reduced in the *Pasteuria*-control in the first and significantly reduced in the second experiment compared to the respective placebo-control treatment ($p<0.01$, Dunnett´s test) (Fig. 5). Results of the two-way ANOVA indicate no differences between experiments, since the two single factors "CsA" and "*Pasteuria*" affected reproduction significantly in both experiments (all $p<0.01$), while their joint effect (CsA**Pasteuria*) on reproduction was additive in both cases as indicated by the non-significant interaction term (Tab. 2). The number of offspring decreased with increasing concentrations of CsA in both experiments. In the absence of *Pasteuria*, reproduction was significantly reduced down to CsA concentrations of 0.003 mg/l (i.e. NOEC of 0.001 mg/l), while in combination with the parasite CsA significantly impacted reproduction only down to the 10-fold higher concentration of 0.032 mg/l (Fig. 5). Yet, the higher variation for the reproduction estimate due to a lower number of *Daphnia* contributing to reproduction hampers a direct comparison and results in the non-significance of the interaction (Table 2). The absence of an interaction between the two stressors indicates that fecundity of *Pasteuria*-challenged, but non-infected hosts is not synergistically impacted by CsA. In consistence with this result, the EC_{10} values estimated for the standard endpoint reproduction did not significantly differ between absence and presence of parasite challenge (overlapping confidence intervals, Tab. 3).

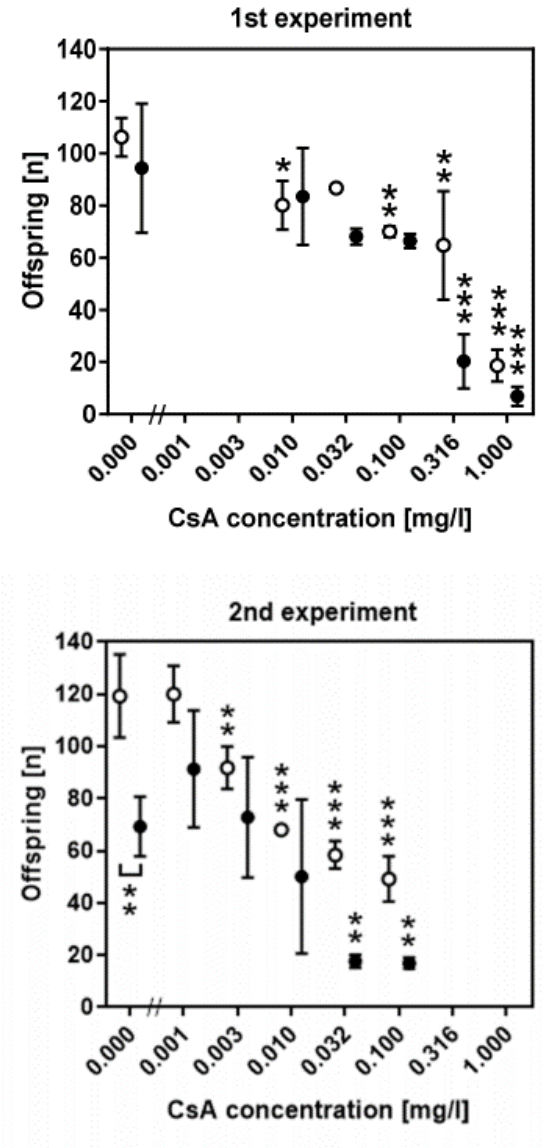

Figure 5: Number of offspring per surviving, non-sterilized *D. magna* until day 21 in dependence of CsA alone (Placebo, white symbols) or in combination with *P. ramosa* challenge (black symbols). Shown are means per treatment with their standard deviations in the first (top) and the second experiment (bottom). Significant differences between each treatment and its respective CsA-free control (placebo or *Pasteuria*) and between controls: * $p<0.05$; ** $p<0.01$; *** $p<0.001$ (Dunnett's test).

3.1.4 Population growth rate (*r*)

The population growth rate was significantly reduced in the *Pasteuria*-control treatments in comparison with the placebo-controls in both experiments ($p<0.05$, Dunnett´s test) (Fig. 6). As revealed by the two-way ANOVA, both single factors "CsA" and "*Pasteuria*" affected the population growth rate significantly in both experiments (all $p<0.001$) (Tab. 2). Since calculation of *r* integrates reproduction, survival as well as *Pasteuria*-induced sterilization of *D. magna*, stronger effects of the combined stressors were observed compared to the endpoint

reproduction. That was obvious in the significant interaction term (CsA**Pasteuria*) in the first test, but not in the second test with lower test concentrations and higher survival rates (Tab. 2). The strongest effect on population growth rate (decrease by 86% compared to the CsA-free *Pasteuria*-treatment) was observed at 1.0 mg/l CsA in the presence of *Pasteuria* (Fig. 6). Effect concentrations determined for *r* were about 2-fold greater with *Pasteuria* challenge compared to placebo treatments (Tab. 3). Based on the relatively broad confidence intervals, however, this difference was not significant.

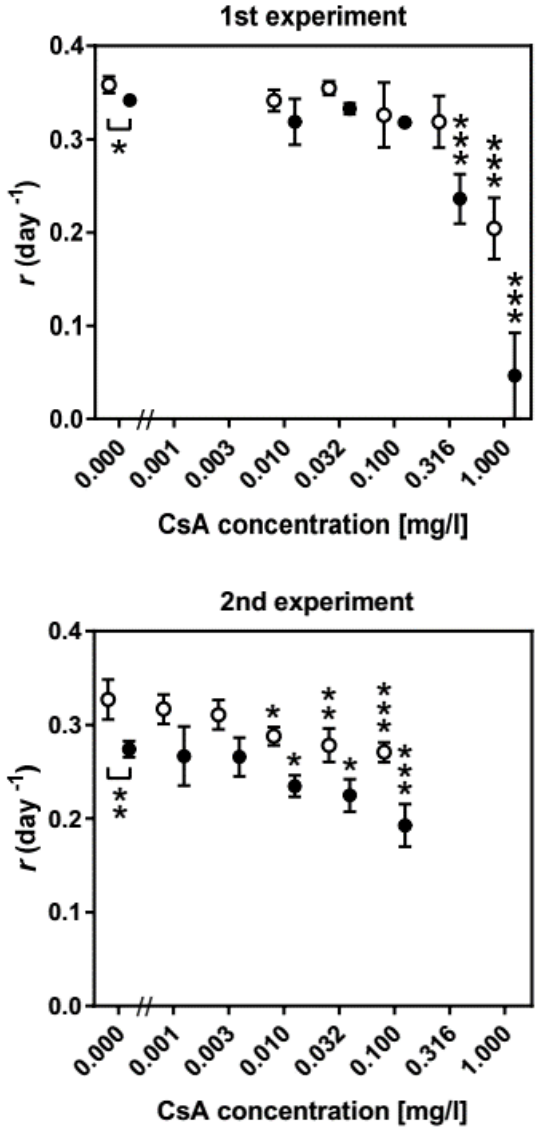

Figure 6: Population growth rate of *D. magna* until day 21 in dependence of CsA alone (Placebo, white symbols) or in combination with *P. ramosa* challenge (black symbols). Shown are means per treatment with their standard deviations in the first (top) and the second experiment (bottom). Significant differences between each treatment and its respective CsA-free control (placebo or *Pasteuria*) and between controls: * $p<0.05$; ** $p<0.01$; *** $p<0.001$ (Dunnett's test).

Table 2: Results of the ANOVA for sterilization at day 10 and day 21, reproduction, and population growth rate.

	1st experiment				2nd experiment			
	DF	MS	F	p	DF	MS	F	p
Sterilization rate d10								
CsA	1	763.4	2.15	0.162	1	1455.1	6.24	0.020
Residuals	16				22			
Sterilization rate d21								
CsA	1	5208.6	24.74	<0.001	1	4054.7	13.43	0.001
Residuals	16	210.6			22	301.9		
Reproduction								
CsA	1	25187.8	85.41	<0.001	1	20944.4	40.13	<0.001
Pasteuria	1	2455.2	8.33	0.007	1	13035.0	24.97	<0.001
CsA x *Pasteuria*	1	82.2	0.28	0.601	1	44.00	0.08	0.773
Residuals	32	294.9			44	521.9		
Population growth rate								
CsA	1	0.217	421.28	<0.001	1	0.022	47.68	<0.001
Pasteuria	1	0.024	46.67	<0.001	1	0.037	82.12	<0.001
CsA x *Pasteuria*	1	0.024	47.04	<0.001	1	0.001	2.09	0.155
Residuals	32	0.001			44	0.000		

Table 3: Determined effect concentrations (EC_{10} and EC_{50}) in the experiments with *D. magna* exposed to CsA with and without additional *P. ramosa* challenge. Results of the two experiments were related to their respective treatment control (placebo or *Pasteuria*), subsequently combined and values are given in mg/l, with 95% CI.

	Placebo	P. *ramosa*
$EC_{50\_survival\_d10}$	>1.0	>1.0
$EC_{50\_survival\_d21}$	0.203 (0.081–0.325)	0.013 (0.007–0.018)
$EC_{50\_sterilization\_d10}$	-	0.542 (0.539–0.545)
$EC_{50\_sterilization\_d21}$	-	0.003 (0–0.006)
$EC_{10\_reproduction}$	0.001 (0–0.002)	0.019 (0.014–0.025)
$EC_{50\_reproduction}$	0.14 (0.045–0.236)	0.059 (0.048–0.07)
EC_{10_r}	0.019 (0.001–0.039)	0.01 (0.003–0.018)

4 Discussion

The present study tested the hypothesis that exposure to an immunosuppressing pharmaceutical increases the virulence of a bacterial pathogen in an invertebrate host. This hypothesis was confirmed by the finding that co-exposure to CsA significantly enhanced mortality and sterilization as well as speed of sterilization of the invertebrate host *D. magna*.

Host survival and *Pasteuria*-induced infection rate were the most sensitive endpoints in detecting immunotoxicity in the present study as shown for example by the estimated $EC_{50\_d21(survival)}$ values, which indicate an about 12-fold greater toxicity of CsA in the presence of *Pasteuria* challenge.

Whereas survival of *D. magna* was synergistically affected by the combination of the biotic and the chemical stressor, *Pasteuria*-challenge alone did not lead to a significantly reduced host survival in comparison to the placebo-controls. This is in concordance with the literature, keeping in mind the exposure duration of 21 days in the present study. *P. ramosa* is a slow-developing parasite, exploiting host resources and thus depends on the living host (Ebert et al., 2004). To ensure successful transmission of mature spores, *P. ramosa* therefore usually expresses low virulence and kills infected daphnids not prior to 40 - 50 days after initial infection (Jensen et al., 2006). This adapted virulence has been demonstrated to be a result of co-evolution in this host-parasite system (Decaestecker et al. 2007). The present study demonstrates that this balanced virulence can be disturbed by exposure to an immunosuppressive substance.

The significantly accelerated sterilization rate of *P. ramosa*-infected *D. magna* after challenge with CsA is one of the most distinct evidences for the immunosuppressive effects of the substance. Speed of sterilization, as seen by the significant effect of CsA on sterilization rate already at day 10, may serve as an indicator for an enhanced virulence as it has been described earlier for an insecticide (Coors et al., 2008; Coors & De Meester, 2008, 2011).

Reproduction, in contrast, was not synergistically impacted in challenged, but finally non-infected hosts. This may indicate that reproduction is no suitable indicator for immunotoxicity in this host-parasite system, because e.g. the host's immune response to the pathogen does not imply immediate costs in terms of reduced reproduction. Yet, it could also be that non-sterilized *Daphnia* did not successfully fight down the parasite, but were simply not infected in the first place, which means that their immune system was in fact not challenged by the parasite. Without using immune-response specific biomarkers it is not possible to distinguish this.

While the toxicity of the immunosuppressant cannot be definitively determined due to the lack of supporting analytical verification, effects on chronic endpoints were observed in the present study consistently down to rather low concentrations. The estimated NOEC for reproduction in absence of parasite challenge as ecotoxicological standard endpoint was 1 µg/l (Fig. 5). The calculation of the predicted environmental concentration (PEC) according to the EMA-guideline (EMA 2006) with default parameters and 250 mg as defined daily dose (DDD) (Whocc.no, 2018) results in a worst case estimate of 1.25 µg/l. Hence, there is no margin of safety between these rough estimates for effect and exposure concentrations. This indicates that a more robust environmental risk assessment is warranted, by refining the PEC and aquiring effects data for other species such as fish.

Comparison of the here derived NOEC to the acute immobilization EC_{50} of 29.6 mg/l (Campos et al. 2014) results in the very high Acute-to-Chronic Ratio (ACR) of 29,600. This is an exceptionally high ACR compared to the ACR around 10 for chemicals acting by narcosis, i.e. a non-specific mode of action (Ahlers et al., 2006). It can be interpreted as strong sign for a specific mode of toxic action of CsA in *D. magna*. The underlying reason would be a change of the mode of toxic action from acute to chronic exposure, i.e. from non-specific baseline toxicity to specific, receptor-mediated toxicity.

In humans, CsA acts on both the adaptive and the innate immune system, suppressing for example the production of cytokines and inhibiting calcineurin pathways, which are part of the innate immune system (García et al., 1998; Tsuda et al., 2012). Cytokines are proteins involved in the proliferation and differentiation of (immune) cells (Lackie, 2010). Calcineurin is an enzyme that stimulates the formation of important inflammatory activators in the nucleus of immune cells like T-lymphocytes and basophils at least in vertebrates. The inhibition of the calcineurin thus leads to a reduced response of the immune system (Matsuda & Koyasu, 2000). The existence of both cytokines and calcineurin is not known for daphnia species, but has been demonstrated in represantatives of higher crustacea (Ottaviani et al., 2003; Chang & Mykles, 2011). Studies with *Galleria mellonella*, showed that lipophorin may be the major CsA-binding protein in insects (Vilcinskas et al., 1997). Again, lipophorin was detected in representatives of the crustacean family (Khan et al., 2018), but no literature is available regarding the existence of lipophorin in *D. magna*. It is assumable, yet remains unclear if above mentioned mechanisms were involved in host immune responses in the present study.

In the innate immune system of vertebrates, which resembles that of invertebrates, CsA acts on the Toll-like receptor (TLR) (Lim et al., 2005). This receptor recognizes fungi or gram-positive bacteria. In *Daphnia pulex*, whose genome was already fully sequenced, the presence of TLR was confirmed by comparison with the genome of *Drosophila melanogaster* (McTaggart et al., 2009). Since *P. ramosa* is a gram-positive bacterium and CsA inhibits TLR, the detection of invading spores is likely to be inhibited by CsA if the TLR is present in *D. magna* and the mechanism is analogous to that of vertebrate organisms.

In general, the defense mechanisms of *D. magna* against *P. ramosa* are not yet fully understand and studies on the molecular mechanisms involved in stress responses of *D. magna* to *Pasteuria* infections are rare so far. However, presence of phagocytic cells has been demonstrated in *D. magna* and phagocytosis was shown to play a role in the defense against *P. ramosa* (Auld et al., 2012). Since Vilcinskas et al., (1999) reported inhibiting effects of CsA on phagocytic cells in the greater wax moth *G. mellonella*, it is assumable that CsA may also have inhibiting effects on phagocytosis in *D. magna*, leading to the observed effects of enhanced host susceptibility to *P. ramosa* in the present study. Another major component of invertebrate innate immune response is the prophenoloxidase-activating (proPO) system, which has been demonstrated in daphnids (Cerenius and Söderhäll, 2004). The proPO system has antimicrobial effects and is involved in the defense against encapsulated microorganisms. During infection the inactive proenzyme proPO is transformed into the active form phenoloxidase (PO), which induces the production of melanin. Activation of proPO catalyzes the formation of quinonoid and cytotoxic oxygen radicals, which play a major role in the defense against pathogens (Söderhäll & Cerenius, 1998). Mucklow and Ebert (2003) found a negative correlation between measured proPO activity and *P. ramosa* infection in experiments with several strains of daphnia. *Daphnia*

clones with increased proPO activity appear to be more resistant to *P. ramosa*. Thus, it may well be that the proPO system plays a role in fighting *P. ramosa*. However, the mechanisms are not yet known, CsA may affect the proPO cascade in *D. magna* and thus lead to the enhanced susceptibility against *P. ramosa.*

The here employed host-pathogen system *Daphnia magna - Pasteuria ramosa* is characterized by a highly specific and distinct infection response and it is well-studied in terms of host and parasite genetics and their interactions in evolutional and ecological contexts (Carius et al. 2001; Luijckx et al., 2011; Ebert et al., 2016). According to results of the present study and in concordance with other studies (e.g. Coors & De Meester, 2008, 2011; Buser et al., 2012; Schlüter-Vorberg et al., 2017), the *Daphnia-Pasteuria* system appears as a promising *host resistance* model for the detection of immunotoxicity in invertebrates. The enhanced virulence of *P. ramosa* is detectable by distinct responses of the host, such as reduced survivorship and accelerated speed and increased rate of sterilization, which are easy-to-measure endpoints. While controlled infections can be successfully achieved (e.g. Coors et al., 2008; De Coninck et al., 2013), considerable host mortality in the *Pasteuria*-only treatment as observed in the present study may be a sign for too strong infection conditions. In addition, it was challenging to achieve the intended 50% host infection rate at test end in absence of the chemical stressor. Hence, more effort in standardization would be required to develop the *Daphnia magna – Pasteuria ramosa* system into a *host resistance assay* that can be used for environmental risk assessments of potentially immunotoxic chemicals.

5 Conclusions

The present study demonstrates that the virulence of the natural *D. magna* parasite *P. ramosa* was enhanced by co-exposure to the immunosuppressant CsA. Enhanced virulence was expressed in terms of reduced host survival and increased host infection rate as well as speed of sterilization in combined treatments. Thus, this implies that CsA impacted the immune system of *D. magna*, and that it would consequently be considered immunotoxic (immunosuppressant) in consistence with results from mammalian *host resistance assays* (Descotes, 2005). In general, the present study provides clear evidence for a suppressed disease resistance in a chemically stressed invertebrate host, highlighting the need of considering relevant factors such as the conjunction of environmental pollutants and pathogens in the environmental risk assessment of anthropogenic pollutants.

Conflict of interest disclosure

The authors declare no competing interests.

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Supplementary information (SI)

to

Impact of an immunosuppressant on the interaction of a bacterial parasite and its invertebrate host

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1. Fitted dose-response curves for the investigated endpoints

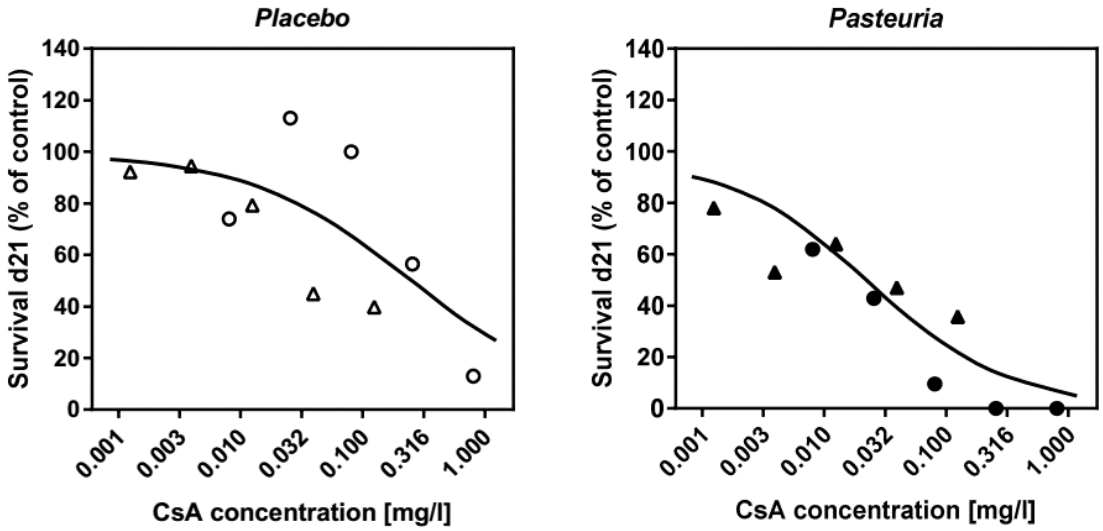

Figure 1: Survival of *D. magna* until day 21 in dependence of CsA alone (Placebo, white symbols) or in combination with *P. ramosa* challenge (black symbols). Shown are means per treatment in % of their respective control in the first and the second experiment (circles and triangles, respectively) fitted by a two-parameter log-logistic model.

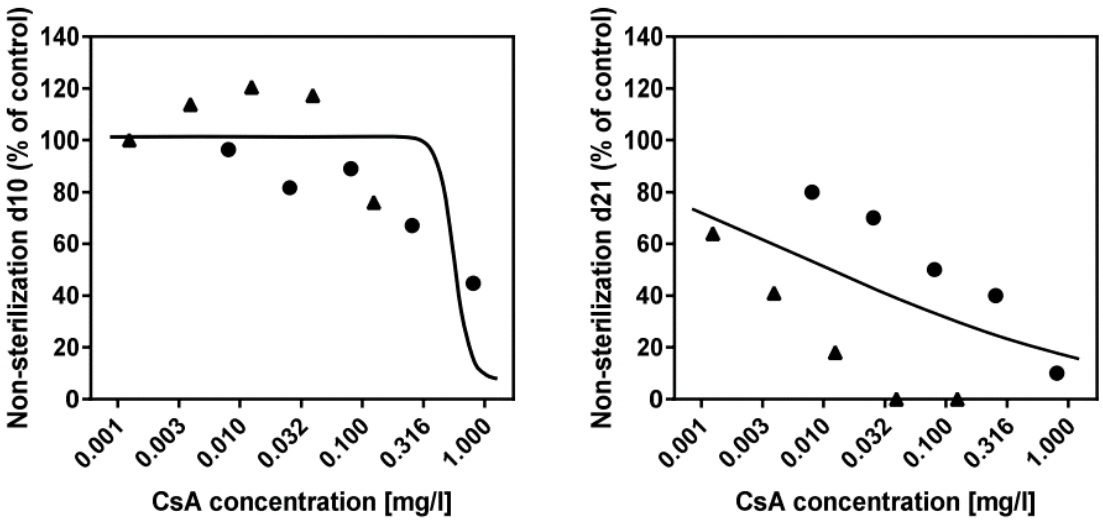

Figure 2: Sterilization rate (given as non-sterilization) of *D. magna* at day 10 (left) and day 21 (right) in dependence of CsA. Shown are means per treatment in % of their respective control in the first and the second experiment (circles and triangles, respectively) fitted by a two-parameter log-logistic model.

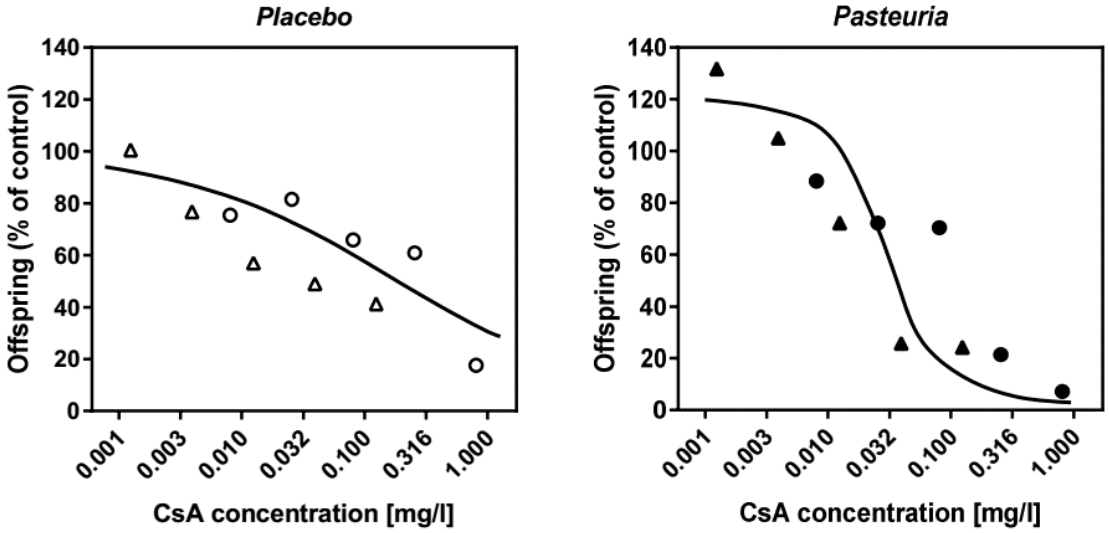

Figure 3: Reproduction of *D. magna* until day 21 in dependence of CsA alone (Placebo, white symbols) or in combination with *P. ramosa* challenge (black symbols). Shown are means per treatment in % of their respective control in the first and the second experiment (circles and triangles, respectively) fitted by a two-parameter log-logistic model.

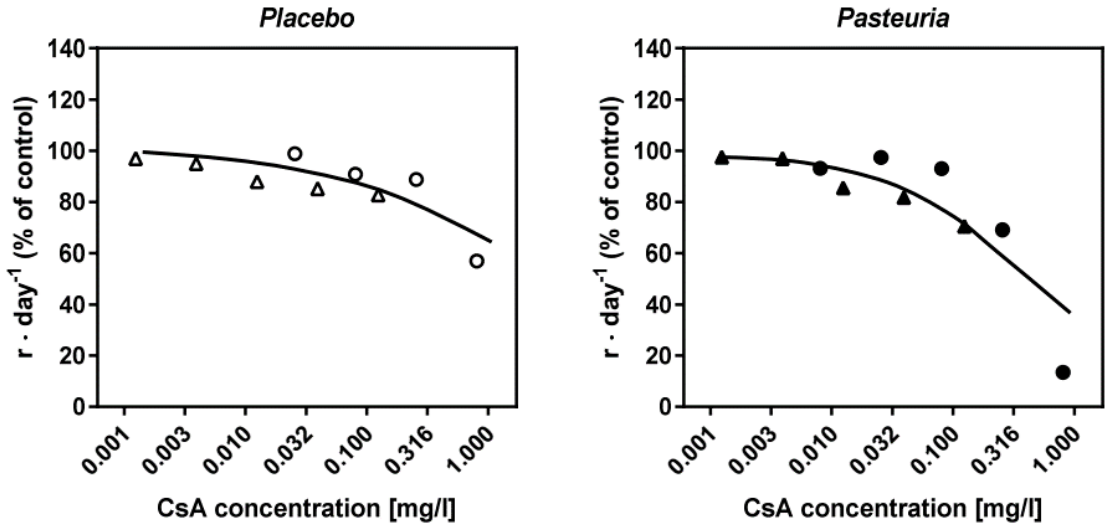

Figure 4: Population growth rate of *D. magna* until day 21 in dependence of CsA alone (Placebo, white symbols) or in combination with *P. ramosa* challenge (black symbols). Shown are means per treatment in % of their respective control in the first and the second experiment (circles and triangles, respectively) fitted by a two-parameter log-logistic model.