Analytical screening for possibly toxic plant secondary metabolites in surface waters by liquid chromatography coupled to high resolution mass spectrometry

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List of Abbreviations

AChE	Acetylcholinesterase inhibition
BD	Bode catchment
BS	Ballegab Skovbaek stream
DI	Direct injection
EC	Effect concentration
ELP	Elster, Luppe and Pleiße catchment
ESI	Electrospray ionization
KM	Kvak Moellebaek stream
LC-HRMS	Liquid chromatography coupled to high resolution mass spectrometry
LVSPE	Large volume solid phase extraction
m/z	Mass-to-charge ratio
MDL	Method detection limit
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
ND	Not detected
NQ	Not quantified
NTS	Non target screening
PAs	Pyrrolizidine alkaloids
PSMs	Plant secondary metabolites
RQ	Risk quotient
RQ	Risk quotient
TTC	Threshold for toxicological concern
VJ	Vejle river

Abstract

A large number of chemicals are constantly introduced to surface water from anthropogenic and natural sources. Although substantial efforts have been made to identify these chemicals (e.g potentially anthropogenic contaminants) in surface waters using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS), a large number of LC-HRMS chemical signals often with high peak intensity are left unidentified. In addition to synthetic chemicals and transformation products, these signals may also represent plant secondary metabolites (PSMs) released from vegetation through various pathways such as leaching, surface run-off and rain sewers or input of litter from vegetation. While this may be considered as a confounding factor in screening of water contaminants, it could also contribute to the cumulative toxic risk of water contamination. However, it is hardly known to what extent these metabolites contribute to the chemical mixture of surface waters. Thus, reducing the number of unknowns in water samples by identifying also PSMs in significant concentrations in surface waters will help to improve monitoring and assessment of water quality potentially impacted by complex mixtures of natural and synthetic compounds. Therefore, the main focus of the present study was to identify the occurrence of PSMs in river waters and explore the link between the presence of vegetation along rivers and detection of their corresponding PSMs in river water.

In order to achieve the goals of the present thesis, two chemical screening approaches, namely, non-target and target screening using LC-HRMS were implemented. (1) Non-target analysis involving a novel approach has been applied to associate unknown peaks of high intensity in LC-HRMS to PSMs from surrounding vegetation by focusing on peaks overlapping between river water and aqueous plant extracts (Annex A1). (2) LC–HRMS target screening in river waters were performed for about 160 PSMs, which were selected from a large phytotoxin database (Annex A2 and A3) considering their expected abundance in the vegetation, their potential mobility, persistence and toxicity in the water cycle and commercial availability of standards.

In non-target screening (Annex A1), a high number of overlapping peaks has been found in between aqueous plant extracts and water from adjacent location, suggesting a

significant impact of vegetation on chemical mixtures detectable in river waters. The chemical structures were assigned for 12 pairs of peaks while several pairs of peaks whose MS/MS spectra matched but no structure suggestion were made by the implemented software tools for retrieving possible chemical structure. Nevertheless, the pairs of peaks with matching spectra represented the same chemical structure. The identified compound belonged to different compound classes such as coumarins, flavonoids besides others. For the identified PSMs individual concentration up to 5 µg/L were measured. The concentration and the number of detected PSMs per sample were correlated with the rain event and vegetation coverage.

Target screening unraveled the occurrence of 33 out of 160 target compounds in river waters (Annex A2 and A3). The identified compounds belonged to different classes such as alkaloids, coumarins, flavonoids, and other compounds. Individual compound concentrations were up to several thousand ng/L with the toxic alkaloids narciclasine and lycorine recording highest maximum concentrations. The neurotoxic alkaloid coniine from poison hemlock was detected at concentrations up to 0.4 µg/L while simple coumarins esculetin and fraxidin occurred at concentrations above 1 µg/L. The occurrence of some PSMs in river water were correlated to the specific vegetation growing along the rivers while the others were linked to a wide range of vegetation. As an example, narciclasine and lycorine was emitted by the dominant plant species from Amaryllidaceae family (e.g. *Galanthus nivalis* (snow drop), *Leucojum vernum* and *Anemone nemorosa*) while intermedine and echimidine were from *Symphytum officinale*. The ubiquitous occurrence of simple coumarins fraxidin, scopoletin and aesculetin could be linked to their presence in a wide range of vegetation.

Due to lack of aquatic toxicity data for the identified PSMs (in both target and non-target) and extremely scarce exposure data, no reliable risk assessment was possible. Alternatively, risk estimation was performed using the threshold for toxicological concern (TTC) concept developed for drinking water contaminants. Many of the identified PSMs exceeded the TTC value (0.1 µg/L) thus caution should be taken when using such surface waters for drinking water abstraction or recreational use.

This thesis provides an overview of the occurrence of PSMs in river water impacted by the massive presence of vegetation. Concentration for many of the identified PSMs are well within the range of those of synthetic environmental contaminants. Thus, this study adds to a series of recent results suggesting that possibly toxic PSMs occur in relevant concentrations in European surface waters and should be considered in monitoring and risk assessment of water resources. Aquatic toxicity data for PSMs are extensively lacking but are required to include these compounds in the assessment of risks to aquatic organisms and for eliminating risks to human health during drinking water production.

Zusammenfassung

Analytisches Screening von möglicherweise toxischen pflanzlichen Sekundärmetaboliten in Oberflächengewässern durch Flüssigchromatographie gekoppelt mit hochauflösender Massenspektrometrie

Pflanzen produzieren im Laufe ihres Lebens eine Vielzahl bioaktiver Verbindungen, die grob in primäre und sekundäre Stoffwechselprodukte eingeteilt werden. Primäre Metaboliten sind für das Wachstum und die Aufrechterhaltung der zellulären Funktion der Pflanze notwendig, während sekundäre Metaboliten für ihr Wachstum und Überleben unwesentlich sind, aber eine wichtige Rolle bei der Abwehr von Pflanzenfressern und anderen Interarten spielen. Das Hauptmerkmal von Sekundärmetaboliten ist ihre Vielfalt in der chemischen Natur, aufgrund derer sie sich vier Hauptklassen zuordnen lassen: Terpene, Phenole, Glukoside und stickstoffhaltige Alkaloide. Manche dieser pflanzlichen Sekundärmetaboliten (PSM) zeigen eine pharmakologische Wirkung, andere hingegen sind toxisch. Beispielsweise stellen Pyrrolizidinalkaloide, die häufig in Pflanzen der Familien Boraginaceae, Asteraceae, Orchidaceae und Fabaceae vorkommen, genotoxische und karzinogene Risiken für Tiere, einschließlich des Menschen dar. Die Furocumarine, die von Pflanzen der Familien Apiaceae und Rutaceae produziert werden, zeigen Antitumor-Wirkungen in einer Vielzahl von Zelltypen. Umgekehrt sind sie potenzielle Photosensibilisatoren, die entweder nach Hautkontakt oder nach Einnahme mit anschließender ultravioletter Sonnenbestrahlung schwere Phytophotodermatitis verursachen können (siehe Einleitung). PSM werden von den sie produzierenden Pflanzen über verschiedene Wege in das Gewässersystem freigesetzt, wie z.B. über Auswaschung, Oberflächenabflüsse und Regenwasserkanäle oder auch den Abbau pflanzlichen Materials. Sobald PSM in das Gewässersystem gelangen, erleiden sie das gleiche Schicksal wie anthropogene Verbindungen. Daher ist ähnlich wie für viele anthropogene Verbindungen das Vorkommen dieser organischen Verbindungen natürlichen Ursprungs in der Umwelt, u.a. in Oberflächengewässern, von wachsender Bedeutung. Das gilt sowohl für aquatische Ökosysteme als auch für den Menschen.

Täglich werden große Mengen an Chemikalien anthropogenen und natürlichen Ursprungs in unsere Oberflächengewässer eingeleitet. Für qualitative und ggf. auch

quantitative Nachweise wird häufig hochauflösende Massenspektrometrie gekoppelt mit Flüssigchromatographie (LC-HRMS) verwendet. Trotz großer getätigter Anstrengungen, die in Oberflächengewässern detektierten Chemikalien (u.a. potentiell anthropogene Schadstoffe) zu identifizieren, gibt es noch immer eine große Anzahl nicht identifizierter Signale – teils mit sehr hoher Intensität. Neben synthetischen Chemikalien und deren Umwandlungsprodukten können diese Signale auch von PSM stammen, die von der Vegetation freigesetzt werden. Dies kann nicht nur als Störfaktor beim Screening von Wasserverschmutzungen betrachtet werden, sondern auch zum kumulativen toxischen Risiko beitragen. Es ist jedoch kaum bekannt, in welchem Ausmaß diese Metabolite zu chemischen Mischungen in Flüssen und anderen Gewässern beitragen. Die Identifizierung von hochkonzentrierten PSM würde helfen, die Überwachung und Bewertung der Wasserqualität zu verbessern, die möglicherweise durch komplexe Mischungen aus natürlichen und synthetischen Verbindungen beeinflusst wird.

Das Hauptaugenmerk der vorliegenden Studie lag daher auf der Identifizierung von in Flüssen vorkommenden und möglicherweise toxischen PSM. Zudem wurde der Zusammenhang zwischen der Vegetation entlang der Flüsse und dem Nachweis der entsprechenden PSM im Flusswasser untersucht. Darüber hinaus wurde auch die Bedeutung von Regenereignissen für das Auftreten und die Konzentration von PSM in Oberflächengewässern untersucht. Die abschließende Risikobewertung wurde aufgrund unzureichender toxikologischer Daten für die nachgewiesenen PSM unter Verwendung des TTC-Konzepts (threshold for toxicological concern) durchgeführt, das für nicht-genotoxische und nicht-karzinogene Schadstoffe in der Trinkwasserkontrolle entwickelt worden ist.

Um der genannten Zielsetzung nachzugehen, wurden im Rahmen dieser Arbeit Wasserproben aus drei Einzugsgebieten entnommen – zwei davon befinden sich im nordwestlichen Teil des Bundeslandes Sachsen (in der Nähe der Stadt Leipzig) und in Sachsen-Anhalt (Bode), Deutschland, während das dritte Einzugsgebiet in der Kommune Vejle (Haraldskaer) in der Region Süddänemark liegt. Die Einzugsgebiete und beprobten Flüsse wurden aufgrund ihrer üppigen Vegetation entlang des Ufers ausgewählt, die möglicherweise „chemische Fingerabdrücke“ der Metaboliten hinterlässt. In den

deutschen Probegebieten wurden 38 Wasserproben während Regenereignissen zur Direktinjektion (DI) in die LC-HRMS entnommen. Diese wurden ergänzt durch 15 Trockenwetterproben (als Kontrolle). In Dänemark wurden 20 weitere Proben mittels großvolumiger Festphasenextraktion (LVSPE) während Regenereignissen entnommen. Darüber hinaus wurden im Laufe der Zeit wiederholt weitere Wasserproben aus den Flüssen der Einzugsgebiete entnommen, einschließlich vor und nach Regenereignissen. Während der Probenahme wurde die Umgebung der beprobten Flüsse in Deutschland hauptsächlich von einkeimblättrigen und knollenförmigen Pflanzen wie Allium ursinum, Anemone nemorosa, Galanthus nivalis und Leucojum vernum sowie anderen Waldbäumen wie Quercus robur, Fraxinus excelsior, Acer pseudoplatanus und Ulmus dominiert. In Dänemark hingegen wurden die Flüsse gespeist durch Wasser von landwirtschaftlich genutzte Flächen mit Gerste, Weizen und Zuckerrüben, aus Wäldern mit hohem Vorkommen von Alnus glutinosa (gemeine Erle), Petasites hybridus (Pestwurz), Symphytum officinale, Urtica dioica und Grasland mit Senecio Jacobaea L (siehe Anhang A1-A3 für Einzelheiten zur Vegetation). Zusätzlich wurden Wasserproben um Pflanzen der angrenzenden Flächen ergänzt (wässrige Pflanzenextrakte), um das Vorkommen ihrer PSM im Flusswasser zu untersuchen.

Für die Identifizierung der PSM in Fließgewässern, wurden zwei chemischen Analyseverfahren angewandt: Non-Target und Target Screening. Beide Verfahren basieren auf den Ergebnissen der LC-HRMS Messungen. Mittels Non-Target Screening wurden unbekannte Peaks von hoher Intensität mit PSM aus der umgebenden Vegetation verknüpft. Hierfür wurden ausschließlich PSM-Peaks betrachtet, die sowohl im Flusswasser als auch in wässrigen Pflanzenextrakten vorkamen. Zudem wurde anhand der Flusswasserproben ein Target Screening basierend auf etwa 160 Pflanzenmetaboliten durchgeführt. Die Target-Substanzen wurden aus einer großen Phytotoxin-Datenbank (Anhang A2 und A3) unter Berücksichtigung ihrer zu erwartenden Abundanz in der Flussvegetation, ihrer potenziellen Mobilität, Persistenz und Toxizität im Wasserkreislauf und der kommerziellen Verfügbarkeit von Standards ausgewählt.

Als Ergebnis des Non-Target Screening (Anhang A1) wurde eine hohe Anzahl sich überlappender Peaks zwischen den wässrigen Pflanzenextrakten und Wasser von

angrenzenden Standorten festgestellt. Dieser Zusammenhang deutet auf einen signifikanten Einfluss der Vegetation auf Chemikalienmischungen in Flüssen hin. Von den ermittelten Peakpaaren konnten insgesamt 12 einer chemischen Struktur zugeordnet werden. Die identifizierten PSM gehören verschiedenen Klassen von Verbindungen an, u.a. den Flavonoiden (und ihren Glukosiden), Cumarinen und Purinnukleobasen, wobei Flavonoide die vorherrschende Klasse sind. Im Allgemeinen enthalten die meisten der identifizierten Metaboliten eine oder mehrere phenolische Gruppen. Diese sind Teil einer Klasse von Verbindungen, die in der Vegetation am häufigsten vorkommen. Für die identifizierten PSM wurden Einzelkonzentrationen von bis zu mehreren µg/L gemessen. Beispielsweise wurden die Flavonoide Hyperosid und Apiin in einer Konzentration von 4 bzw. 5 µg/L nachgewiesen. Konzentration und Anzahl der einzelnen nachgewiesenen PSM variieren in Wasserproben, die zwar am selben Ort jedoch an unterschiedlichen Regenereignistagen genommen wurden. Die Annahme, dass diese Variationen an die Intensität des Regens während der Probenahme gekoppelt sind, wird durch den fehlenden Nachweis von PSM in den Trockenwetterproben unterstützt (Anhang A1). Auch die Vegetationsbedeckung könnte einen Einfluss auf die Konzentration von Metaboliten haben. Ein Hinweis hierfür ist der Nachweis von bis zu 5 µg/L Apiin in Wasserproben, die bei weniger intensivem Regen entnommen wurden, am Ort der Probenahme jedoch eine massive Präsenz von Digitalis purpurea festgestellt wurde.

Darüber hinaus wurden noch weitere Peakpaare ermittelt, deren MS/MS-Spektren übereinstimmten. Folglich handelt es sich hierbei um dieselben chemischen Strukturen. Eine eindeutige Zuordnung war jedoch nicht möglich. Das Auftreten dieser nicht identifizierten Peaks, die zu den bestätigten Peaks hinzugezählt wurden, bezeugt den Einfluss der entlang von Flüssen wachsenden Vegetation auf die chemische Zusammensetzung des Flusswassers. Unsere Ergebnisse zeigen also umfassend, wie bestimmte Metaboliten oder Metabolitenklassen mit dem Vegetationsvorkommen im Einzugsgebiet und der Jahreszeit korrelieren.

Im Target Screening der Flusswasserproben wurden 33 der ca. 160 untersuchten Substanzen nachgewiesen. (Anhang A2 und A3). Die identifizierten Verbindungen gehören zu verschiedenen Klassen wie u.a. den Alkaloiden, Cumarinen und Flavonoiden,

wobei erstere die am häufigsten vorkommende Klasse ist. Zu den identifizierten Verbindungen gehören die Pyrrolizidinalkaloide Echimidin, Intermedin und ihre N-Oxide, die Flavonoide Daidzein und Rutin sowie die Cumarine Psoralen und Fraxetin. Die Konzentrationen einzelner Verbindungen betrugen bis zu mehreren hundert ng/L, wobei die toxischen Alkaloide Narciclasin und Lycorin die höchsten Maximalkonzentrationen aufwiesen. Auch das neurotoxische Alkaloid Coniin des Giftschierlings gehörte zu den nachgewiesenen Target-Substanzen. Das Vorkommen einiger PSM im Flusswasser korreliert mit der spezifischen Vegetation entlang der Flüsse, während die anderen mit einem breiten Spektrum an Pflanzen in Verbindung gebracht werden. Zum Beispiel Narciclasin und Lycorin, die von den dominanten Pflanzenarten aus der Familie der Amaryllidaceae (z.B. Galanthus nivalis (Schneeglöckchen), Leucojum vernum und Anemone nemorosa) emittiert werden, während Intermedin und Echimidin aus Symphytum officinale stammen. Das ubiquitäre Vorkommen der einfachen Cumarine Fraxidin, Scopoletin und Aesculetin konnte mit ihrem Vorhandensein in einem breiten Spektrum an Pflanzen in Verbindung gebracht werden.

Einige dieser Verbindungen wie Piperin, Daidzein und Nikotin sind natürlich vorkommende Verbindungen, die von manchen Pflanzen selbst produziert werden. Da in dem untersuchten Einzugsgebiet jedoch keine dieser Pflanzen vorkommen, ist der Eintrag dieser Verbindungen in das Flusswasser hier sehr wahrscheinlich auf menschliche Aktivitäten zurückzuführen. Die genannten PSM werden vom Menschen in großem Umfang konsumiert und stehen in Zusammenhang mit Tabak (Nikotin), Sojabohnen (Daidzein) und Lebensmittelaromen (Piperin). Sie sind ein Indiz für mögliche Emissionen durch Siedlungsabfälle oder Deponiesickerwasser (siehe Einleitung). Mangels geeigneter Überwachungstechniken zum Vorkommen und Entfernen in Kläranlagen könnten solche Verbindungen zudem über Kläranlagenabwasser in die anschließenden Gewässer gelangen.

Wie von mehreren Forschern berichtet, zeigten die im Rahmen dieser Doktorarbeit identifizierten PSM verschiedene pharmakologische Wirkungen wie antibiotische, antimykotische, antivirale, antioxidative und entzündungshemmende Wirkungen (siehe Einleitung). Beispielsweise zeigen Lycorin und Hyperosid eine Acetylcholinesterase-

Inhibitionswirkung, während Coniin als nikotinischer Acetylcholin-Rezeptorantagonist wirkt, was zu einer Hemmung des Nervensystems führen kann, und schließlich zum Tod führt (Anhang A2 und A3). Epidemiologische Daten deuten darauf hin, dass eine relativ hohe ernährungsbedingte Exposition gegenüber den Furocumarinen Bergapten und Psoralen auch das Hautkrebsrisiko erhöhen kann (Anhang A2).

Die durchgeführte Risikobewertung wurde auf der Grundlage von TTC-Werten aus der Trinkwasserüberwachung durchgeführt, da für unbehandeltes (Oberflächen-) Wasser keine TTC-Werte verfügbar sind. Infolgedessen überschritten viele der identifizierten PSM den TTC-Wert, weshalb Vorsicht geboten ist, wenn solche Oberflächengewässer für die Trinkwasserentnahme oder den Freizeitgebrauch verwendet werden. Da in den einzelnen TTC-Werten keine Mischungseffekte berücksichtigt sind, wurden additiven Risikoquotienten (RQs) bestimmt (Anhang A1 und A2), aus denen sich häufig Mischungs-RQs von über 0,1 ergaben. Das überschreiten dieses Wertes deutet darauf hin, dass das Auftreten von PSM in den Mischungen möglicherweise ein Risiko sowohl für Wasserlebewesen als auch für den Menschen darstellt. Toxische Risiken durch einzelne PSM und deren Mischungen sowie ein Beitrag zur Gesamttoxizität von Oberflächengewässern können daher nicht ausgeschlossen werden, und machen zusätzliche Anstrengungen bei der Erfassung von Gefahren erforderlich.

In dieser Arbeit wurde mittels LC-HRMS-basierten Non-Target und Target Screening Verfahren das Auftreten von PSM in Flussgewässern untersucht. Dabei wurden verschiedene PSM nachgewiesen (Anhang A1-A3), die zu den Verbindungsklassen der Alkaloide, Cumarine und Flavonoide gehören und zum Teil häufig vorkommen. In vielen Fällen überstiegen die Konzentrationen dieser Verbindungen, von denen bekannt ist, dass sie eine erhebliche biologische Aktivität aufweisen, die Konzentrationen vieler anthropogener Chemikalien in Oberflächengewässern. So wurden beispielsweise die toxischen Alkaloide Lycorin und Narciclasin in Konzentrationen von 2,3 bzw. 3,4 µg/L nachgewiesen, für Apiin wurde sogar ein Wert von 5 µg/L ermittelt. Es könnte daher sein, dass die in Flüssen nachgewiesenen PSM-Konzentrationen schädliche Auswirkungen auf Wasserlebewesen und den Menschen haben. Obwohl dieser Befund nicht unbedingt auf ein toxisches Risiko für Wasserorganismen hindeutet, kann ein erheblicher Beitrag

zur Mischtoxizität durch die relativ hohen Konzentrationen nicht ausgeschlossen werden. Da die Kenntnisse über das Auftreten und die Auswirkungen von natürlich vorkommenden Verbindungen in der Umwelt begrenzt sind, werden letztere bislang nicht in Umweltüberwachungsprogramme einbezogen – im Gegensatz zu Verbindungen anthropogenen Ursprungs. Die vorliegende Studie ist daher ein erster Schritt, um existierenden Wissenslücken in diesem Bereich zu schließen, wobei die identifizierten Verbindungen nur die Spitze des Eisbergs von möglicherweise toxischen PSM in Wasserressourcen darstellt. Es wird daher empfohlen, PSM bei der zukünftigen Überwachung und Risikobewertung zu berücksichtigen. Sie sollten zudem in Zusammenhang mit der Entnahme von Trinkwasser betrachtet werden. Ein potentielles Risiko besteht insbesondere während Regenereignissen, die den Eintrag von PSM in Oberflächengewässer fördern. Das massive Auftreten toxischer Pflanzen zu bestimmten Jahreszeiten kann nicht ausgeschlossen werden. Informationen über aquatische Toxizität von SPM sowie Daten zur Exposition fehlen weitgehend, sind aber erforderlich, um eine zuverlässige Risikobewertung und Priorisierung von PSM durchzuführen. Daher sollten PSM zunehmend in die chemische Überwachung von Oberflächengewässern einbezogen werden, um in größerem Maßstab Expositionsdaten zu erhalten, ergänzt durch Toxizitätstests von Verbindungen, die häufig oder in hohen Konzentrationen auftreten.

Chapter One

General Introduction

1.1 Naturally occurring toxic compounds

A multitude of diverse organisms and biocoenosises exist in the environment, which have different appearance and way or style of life. Ecosystem functioning reflects the collective life activities of these organisms and the effects of their activities (e.g. feeding, growing, moving and excreting waste) on the physical and chemical conditions of their environment (Wink 2010; Petersen et al. 2020). Thus, a functioning ecosystem exhibits biological and chemical activities characteristic for its type (Naeem et al. 1999; Wink 2010). In the ecosystem, besides individual differences, all living organisms have their own way of absorbing, processing and secreting substances to and from the environment (Petersen et al. 2020). One way of such communication with the surrounding environment is through the release of their metabolites (Wink 2010), which are toxic to other organisms (and are called natural toxins). In this way, nature offers a wide ranges of chemical compounds from various sources such as plants, fungi, algae, bacteria and marine organisms. These compounds (natural toxins) are bioactive compounds produced by living organisms (Bucheli 2014), which are not toxic to the producing organisms but cause adverse effects on other creatures, including humans when consumed and/or used (Fletcher and Netzel 2020). Some of them are extremely poisonous products of the metabolism (independently of their nature) of living organisms. They serve the producing organisms as a protection from predators or as a tool for hunting and killing prey. Thus, their use in wildlife is part of the passive or active struggle for survival in certain species (Pitschmann 2014). Natural toxins consist of a diverse chemical structure with a wide array of physical-chemical and physiological effects that are as manifold as their chemical structure (Klaschka 2015).

Engaging chemical compounds as defensive agents is widespread among plants, animals, and micro-organisms (Saporito et al. 2012). Living organisms develop a variety of adaption ways for feeding and defense, which are the basic aspects of life. For instance, reptiles (e.g. snakes, few lizards), insects (e.g. spiders, bees), sea creatures (e.g. cone snails and octopuses) produce and use toxic compounds for catching or trapping the prey and also for self-defense to protect themselves from getting preyed (Mohanty et al. 2016). In addition to defensive chemicals that are biosynthesized

endogenously, many organisms sequester defensive chemicals from environmental sources.

1.2 Natural toxins and humans

Human beings use natural toxins for different purposes by relying on their observed and special effects on living organisms (without understanding their principle). As an example, they treat different diseases, to increase resistance to mental and physical fatigue, and thus reduce feeling of hunger, for magic and religious rituals, for hunting animals, for protection from troublesome insects, and from dangerous animals (Ji et al. 2009; Kapoor 2010; Dias et al. 2012; Pitschmann and Hon 2016). The application of natural toxins to commit suicide, murder as well as to wage war have also been reported (Pitschmann and Hon 2016; Pitschmann 2014). For instance, plant extracts such as the juice of *Derris elliptica* and the extracts from plants of the genus *Croton* with ability to exert dazing effects were used for fishing by water poisoning. A variety of chemicals found in these plants stun fish when the compounds pass through the gills or are ingested. The fish then floats to the surface for easy capture (Jones 2007). The techniques of using diverse natural materials were also used in armed conflicts and wars. In ancient Greece, the river Pleistos, the source of drinking water for the city Kirrha, was poisoned by military troops of the League of Delphi with extracts from the hellebore (*Helleborus* spp.), which contains a number of toxins with cardioactive and spastic effects (hellebrin, ranunculin). In a war of Florence and allies against Verona in the 14th century, a drinking water source was poisoned with hemlock (*Conium maculatum*) (Pitschmann and Hon 2016). At the Middle Age (11th century), the juice from *Atropa belladonna* containing tropane alkaloids were used by the Scottish troops to poison food in the encampment of the invading Norwegian army (Hesse 2002; Pitschmann and Hon 2016).

Throughout human history, besides their poisoning effects, the importance of these bioactive compounds for medicine and health has been enormous (Krause and Tobin 2013). For instance, the earliest ancestors chewed herbs containing bioactive compounds to relieve pains and wrapped leaves around the wound to improve the healing (Ji et al. 2009). Nowadays, the bioactive compounds are extremely productive source for new medicines in all cultures and continue to deliver a great variety of structural templates to

discover and develop the final drug entity (Newman and Cragg 2016; Ji et al. 2009). They have been the single most productive and successful source of leads for the development of potential drugs (Harvey 2008; Dias et al. 2012) and have often been the sole means to treat diseases, illnesses and injuries (Ji et al. 2009). Majority of all drugs in the market are either directly produced from natural compounds or derived from their novel chemical structure, which may not necessarily represent active ingredients in their final form (Krause and Tobin 2013). For example, in the area of cancer (from 1940s to 2014), of the 175 small molecules approved as potential drug 75% are other than synthetic with 49% actually being either natural compounds or directly derived therefrom (Newman and Cragg 2016). Thus, naturally-derived products constitute an extremely important resource for global pharmaceutical companies working on the development of new medicines (Harvey 2008).

1.3 Toxic effects of bioactive compounds

Although the majority of bioactive compounds are safe and used in our daily life activities, they are not without risk. Since they are not extensively and systematically studied, the information regarding side effects and risks of toxicity is often lacking. It is incorrect to believe that any product, because it is considered to come from a natural source, is automatically safe (Molyneux et al. 2007). For instance, solanine, isolable from the European black nightshade (*Solanum nigrum*), is highly toxic, even deadly (Smith 2013; Omayio et al. 2016). A sudden death in animals and occasionally in humans, especially children, was reported upon consumption of English yew (*Taxus baccata* L., Taxaceae), a poisonous plant containing toxic compounds (taxine alkaloids) (Wilson et al. 2001; Molyneux et al. 2007). In some cases, the adverse health effects caused by naturally occurring toxic compounds, such as indospicine, are not limited to primarily exposed organisms (e.g livestock), and rather carried through food chain and might cause secondary poisoning in animals consuming the livestock (Fletcher and Netzel 2020).

Some of the bioactive compounds are hepatotoxic, carcinogenic, genotoxic, teratogenic and sometimes pneumotoxic (Wiedenfeld and Edgar 2011). On the basis of their origin, van Egmond classified the naturally occurring toxic compounds (natural toxins) into five main categories, namely: bioactive compounds produced by fungi (mycotoxins), bacteria

(bacterial toxins), algae (phycotoxins), animals (zootoxins) and plants (toxic phytochemicals or phytotoxins) (van Egmond 2004). The occurrence of these natural toxins in the environment is a growing concern to aquatic life and human health. Thus, in order to get an overview, the harmful effects caused by selected natural toxins from various living organisms are discussed below.

Poisonous animals accumulate toxic compounds (zootoxins) in their tissues, resulting in toxic exposure for those who would dare to attack them. Exposure to these compounds is not uncommon due to profusion of toxic animals in the environment (Gwaltney-Brant 2011; Mohanty et al. 2016). Since variations in toxicity of zootoxins are caused by different factors such as age, sex, nutritional status, season, geographic location and toxin composition, not all exposures to toxic animals result in toxicosis (Gwaltney-Brant 2011). Mohanty et al 2016 reported a brief overview of zootoxins from different animals along with their mechanisms of action (see Table 1).

Frogs produce and release poisonous alkaloids (e.g. pumiliotoxins, histrionicotoxins, gephyrotoxins) to defend from predators and/or microorganisms. Over 800 alkaloids, belonging to different structural classes, have been identified in several lineages of poison frogs worldwide (Saporito et al. 2009). Studies suggested that many of the alkaloids are sequestered directly from a natural diet of alkaloid-containing mites, ants, beetles, and millipedes (Saporito et al. 2009; Saporito et al. 2012). Among the alkaloids, batrachotoxin is one of the most toxic alkaloid poisons known. It causes death due to its effect on sodium ion channels in the body. It binds to these channels and jams them open, interfering with nerve transmission and causing muscles to contract. Ultimately, this leads to heart palpitation, then cardiac arrest and death (Cataldi 2016; Saporito et al. 2012; Jeckel et al. 2015).

Table 1: Zootoxins and their mechanism of action (Mohanty et al. 2016).

Animal	**Zootoxins/chemical compound**	**Mechanism of action**	**References**
Jararacussu (*Bothrops jararacussu*)	Phospholipase A2 (PLA2)	Decreases sarcoplasmic Ca2+-ATPase	(Ayres et al. 2015)
Black mamba (Dendroaspis polylepis)	Calciseptine (CAS)	Inactivate L type Ca channel	(Moeller et al. 2012)
Australian tiger snake (Notechis scutatus)	Notexin (Ntx)	Promotes the enzymatic hydrolysis of sarcolemmal phospholipids which results in membrane damage and Ca2+ influx	(Dixon and Harris 1996)
Cobra (Naja atra)	Cobrotoxin (CBTX)	Post-synaptic non-depolarising block	(Šribar et al. 2003)
Krait (Bungarus candidus)	Candoxin (CDX)	Bind to post-synaptic muscle nAChRs produces reversible, non-depolarising block	(Nirthanan et al. 2003)
Russell's viper (Daboia russelii)	Viperotoxin-F (RV-4/RV-7)Pre-synaptic block		(Hodgson and Wickramaratna 2002)
Mamba (Dendroaspis angusticeps)	Calcicludine (CaC)*	Muscarinic effects by binding to muscarinic AChRs and also inactivate L type Ca channel	(Rajagopalan et al. 2009; Moeller et al. 2012)
Rattle snake (Crotalus durissus)	Phospholipase A2 (PLA2)	Post-synaptic effect by desensitization of nAChR	(Doley and Kini 2009; Sampaio et al. 2010)
Funnel web spiders (Atrax robustus)	Robustoxin/d-Atracotoxin	Induces spontaneous, repetitive firing and prolongation (d-ACTX)of action potentials, prolonged acetylcholine releasefrom both somatic and autonomic nerve endings	(Gupta 2007)
Widow-spider (Latrodectus mactans)	α-latrotoxin (α -LTX)	α -LTX interacts with neurexins and latrophilins on the neuronal membrane, induces pore formation on themembrane, causes exocytosis, followed by massive release and then depletion of acetylcholine andnorepinephrine at postganglionic sympathetic synapses	(Gupta 2007)
Brown recluse spider (Loxesceles reclusa)	Sphingomyelinase D (SMASED)	Stimulates cytotaxis atthe site of envenomation,) inactivates serum hemolytic complement leadingto intravascular coagulation, occlusion of smallcapillaries, tissue necrosis, systemic depletion ofclotting factors (VII, IX, XI, XII) and platelet activation	(Gupta 2007)
Trinidad tarantura (Psalmopoeus cambridgei)	Psalmotoxin (PcTx1)	Causes desensitization of ASIC1 (Acid Sensing Ion Channel 1)	(Escoubas et al. 2000)
Deathstalker scorpion/ Israeli yellow scorpion (Leiurus quinquestriatus hebraeus)	Chlorotoxin (CTX), Charybdotoxin (CHTX), Scyllatoxin, Agitoxins (AgTx) Type I, II, II	Increases Ca influx into cardiocytes through L-type Ca channels, inhibits the chloride ion channel)	(Arie-Saadia et al. 1996; Soroceanu et al. 1998)

Giant forest scorpions (Heterometrus fulvipes)	κ-Hefutoxin 1 (Heteroscorpine-1)	Inhibits Kv1.2, Kv1.3 and slows down the activation of Kv1.3	(Meves 2008)
Wasp	Mastoparan (MAS)	Acts as a nonspecific secretagogue primarily involves exocytosis, causes histamine release from mast cells, serotonin and catecholamine release from platelets and chromaffin cells, prolactin release from anterior pituitary respectively, inhibits K_{ATP} both vascular and smooth muscle cells	(Eddlestone et al. 1995)
Honey bee venom (Apis mellifera)	Apamin (APA) Melittin (MLT)	Inhibits SK2, SK3(small conductance calcium channels) Act as cell membrane lytic factor, inhibits protein kinase C, Ca^{2+}/calmodulin-dependent protein kinaseII, myosin light chain kinase and Na^{+}/K^{+}-ATPase	(Santos-Torres et al. 2011; Yang and Carrasquer 1997)
Sea anemone (Stichodactyla gigantea)	Gigantoxin I (epidermal growth factor -like toxin), II, III	Activate TRPV1 indirectly pathway involving ECF receptor via PLA2 and arachidonic acid	(Chen et al. 2002; Cuypers et al. 2011)
Striped blister beetle (Epicauta vittata)	Cantharidin	Inhibits protein phosphatase 2A, resulting in disruption of signal transduction and cell metabolism	(Stair and Plumlee 2004)
Fireflies (Photinus spp.)	Lucibufagins (LBG)	Inhibit sodium–potassium ATPase activity in the myocardial cell membrane	(Brubacher et al. 1999)
Red imported fire (Solenopsis invicta)	Solenopsins and piperidine	Cytotoxic, hemolytic, fungicidal, insecticidal and ant bactericidal properties	(Gupta 2007)
Toad (Bufo marinus)	Bufogenins	Inhibit sodium-potassium ATPase	(Gupta 2007)
Gila monsters (Helodermasuspectum)	Gilatoxin (GTX) Helodermin, Helospectin I and II	Lethal factor, kallikrein like activity, pain, hypotension Vasodilation, hypotension	(Gupta 2007; Grundemar and Högestätt 1990)
Australian paralysis tick (Ixodes holocyclus)	Holocyclotoxin	Inhibits acetylcholine release at the neuromuscular junction	(Grattan-Smith et al. 1997)

Marine zootoxins are naturally occurring poisons derived from marine organisms. These compounds are mainly produced by toxicogenic algae, cyanobacteria and bacteria, which are common food of marine animals (certain species of fish, crustaceans, and molluscs) (Pitschmann and Hon 2016). They are non-protein toxic substances (e.g., domoic acid, gonyautoxins, ciguatera) from dinoflagellates or algae that accumulate in the tissues of marine organisms; cnidarian (jellyfish, anemones) toxins, echinoderm (starfish, sea stars) toxins, mollusk (cone shells, octopus) toxins and fish toxins (venoms and poisons) (Bagnis et al. 1970; Camacho et al. 2007). For instance, following exposure to the domoic acid, the acute neurologic dysfunction, degenerative cardiomyopathy and reproductive failure in California sea lions (*Zalophus californianus*) were reported. It also associated with out-breaks of amnesiac shellfish poisoning in humans and with deaths of a variety of sea birds and mammals (Gwaltney-Brant 2011).

Amatoxins are a group of highly toxic peptides found in several species of mushrooms, including *Amanita phalloides, Amanita virosa, Amanita bisporigera, Amanita ocreata, Amanita verna, Galerina autumnalis, Galerina marginata,* and some species of *Lepiota and Conocybe* (Mas 2005; Wieland et al. 1978). The cyclic peptide amatoxins, among the most potent mushroom toxins known, are a significant cause of acute fulminant liver failure (Horowitz and Moss 2020). About ninety percent of deaths caused by ingestions of mushroom are associated with amatoxin, which primarily causes death through the process of fulminant hepatic failure secondary to liver parenchymal necrosis (Allen et al. 2012). In Western Europe, 50-100 fatal cases associated with amatoxins were reported yearly (Mas 2005). Figure 1 displays the three phases of amatoxin toxicity following ingestion.

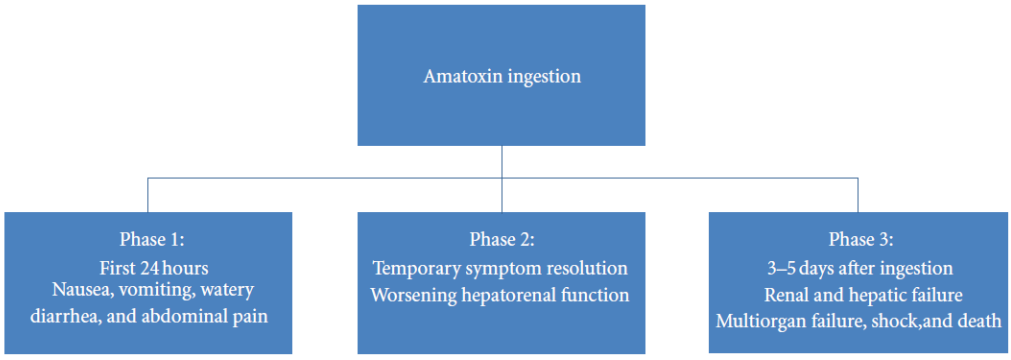

Figure 1. Phases of amatoxin ingestion (Allen et al. 2012).

The fungi (*Aspergillus flavus* and *Aspergillus parasiticus*) producing aflatoxins are commonly found in human food and animal feed (Gupta 2011; Singh et al. 2014). Aflatoxins causes deleterious effects on the reproductive and developmental systems, such as sexual maturation, growth and maturation of the follicles, levels of hormones, gestation and growth of human fetus (Gupta 2011; Mahato et al. 2019). Following oral, inhalative or dermal exposure, aflatoxins exhibited various toxicological effects in humans and animals. Some of the effects due to exposure include an acute illness, followed by death – usually through liver cirrhosis; nutritional and immunologic consequences due to chronic sublethal doses; and all doses have a cumulative effect and the risk of cancer. In addition to dose, duration of exposure and environmental factors, the toxicity of aflatoxin can vary between species, within the same species, age and gender (Gwaltney-Brant 2011). Aflatoxins are mutagenic, carcinogenic, teratogenic and immunosuppressive (Gupta 2011).

Plants and other sessile organisms, which can-not run away in case of danger or which do not have an immune system to combat pathogens, synthesize an enormous variety of low molecular weight compounds known as plant secondary metabolites (PSMs) (Wink 2010; Croteau et al. 2000). These compounds are a wide array of bioactive substances that are vital for the fitness of a plant producing them. Due to their diversity, many PSMs are currently used in so many ways in biotechnology, pharmacy, medicine and agriculture (Wink 2010). PSMs often interfere with more than a single molecular target (multi-target substances), which is advantageous for the producer, as a toxin might be more efficient if it eliminated more than one target. Furthermore, PSMs are always as appearing in mixtures of several substances, often from different classes; e.g. polyphenolics are often accompanied by terpenoids. As a consequence, it will be more difficult for a herbivore or microbe to develop resistance to such a cocktail, as concomitant resistance at several targets would be required (Wink 2008). These mixtures are even more powerful as means of defense and protection than mono-target substances (Wink 2010).

1.4 Natural toxins and the aquatic environment

For the last several decades, the potential contamination effects of anthropogenic compounds on the aquatic system have been extensively investigated. However, little or

no attention have been given to naturally occurring compounds. This could be due to the fact that either compounds of natural origin are mainly considered as safe or that their adverse effects to living organism, specifically aquatic life, are not well communicated (or in some cases that the adverse effects are minimal and negligible). Natural compounds are produced by different kingdoms of life for a specific purpose in the producing organism (Bucheli 2014). Many of them are synthesized by the organisms under specific circumstances to serve different purposes in the environment. Thus, they could be released to the environment in different ways depending on the nature of the stressor or predator. For instance, animals exert their toxic effect when their tissues come into contact with another organism, usually via oral contact and/or when a venomous animal intentionally delivers its toxin to the target animal (e.g., bite or sting) (Gwaltney-Brant 2011). Similarly, plant species exert/release their toxic effects through various ways such as volatilization (wind), root exudate, degradation, and rain sewers, leaching and washing though surface run-off. In this ways the compounds are released into the environment and thus into the aquatic system. Once they reach the aquatic system, they can be of concern for aquatic life and human health, if the water is used for drinking water abstraction. However, the occurrence of these compounds in the aquatic system is hardly explored, which is the main focus of these thesis – specifically compounds of plant origin (PSMs).

These concerns have been addressed in the recently (in 2017) launched European wide project NaToxAq (Natural Toxins and Drinking Water Quality – From Source to Tap),funded by the European Union under Horizon 2020. The focus of the project are natural toxins – a large group of emerging contaminants with unknown impact on drinking water resources and also on aquatic life (https://cordis.europa.eu/project/id/722493). The challenge of natural toxins is addressed by the concerted work of 16 PhD researchers within 4 scientific work packages comprising the origin, distribution, fate and remediation of natural toxins. Thus, as part of this big project, the current thesis contributed on the fate and distribution of PSMs in European surface waters. Consequently, PSMs will be described and addressed in detail in the proceeding sections.

As for anthropogenic compounds, the occurrence of natural compounds (i.e. PSMs) in surface water is of growing concern to aquatic life and humans (van Egmond 2004; Bucheli 2014; Hoerger et al. 2009a). There are several reasons why their environmental occurrence, specifically in the aquatic system, needs to be assessed:

- The emission of PSMs to surface water could be higher from the area covered with massive presence of vegetation such as agricultural lands, forests and grasses.
- New PSMs, not previously present in the area, might be released from invasive plant species.
- Like the many anthropogenic compounds, the occurrence of PSMs in the aquatic environment might pose a risk, some even in minimal quantity, for both aquatic life and humans if the water is used for human consumption or recreational purpose.
- In order to fully evaluate the ecotoxicological effects of aquatic contaminants, the whole chemical composition from anthropogenic and natural origin, should be characterized. This would allow a better understanding of the contribution of PSMs, besides anthropogenic contaminants, for the mixture toxicity effect as well as background stress/toxicity to ecosystems exerted by the presence of such compounds.

1.5 Emission and fate of PSMs in the aquatic ecosystem

The quality of surface water varies considerably due to upstream activities, varying discharge volumes, and flow properties from both anthropogenic and naturally impacted environment. As rivers often flow through several countries, upstream activities and vegetation might influence water quality in other countries downstream, making surface water quality a transboundary issue (Houtman 2010). And also the chemical composition of a river or stream changes during this flow from the source to the mouth. In addition to synthetic compounds introduced by all kinds of human activities, such as agriculture, shipping, industry, and use of chemicals in households (Houtman 2010), river water may contain several naturally occurring organic compounds (i.e PSMs) released from vegetation (Günthardt et al. 2020; Hama and Strobel 2020; Hoerger et al. 2009b; van Egmond 2004). Although PSMs are produced by a plant to function specific purposes, they are also found in products that we use for various aspects for improving our quality

of daily life, ranging from food production and health protection to transport and heavy industry. At some point in their lifetime, these chemicals can enter the water cycle, whether by deliberate discharge after wastewater treatment or as a result of a natural phenomenon (EEA 2018; Al-Shatti et al. 2014). Naturally, PSMs are mainly released into river water through four pathways (Figure 2): (i) volatilization and diffusion away from plant tissues by wind or rain, (ii) leaching of above-ground plant material, (iii) exudation from plant roots and (iv) litter decomposition (Chomel et al. 2016; Al-Shatti et al. 2014; Bucheli 2014). In most cases, rain is the main means of transport of PSMs from source to the receiving river water (Hama and Strobel 2020; Clauson-Kaas et al. 2016)

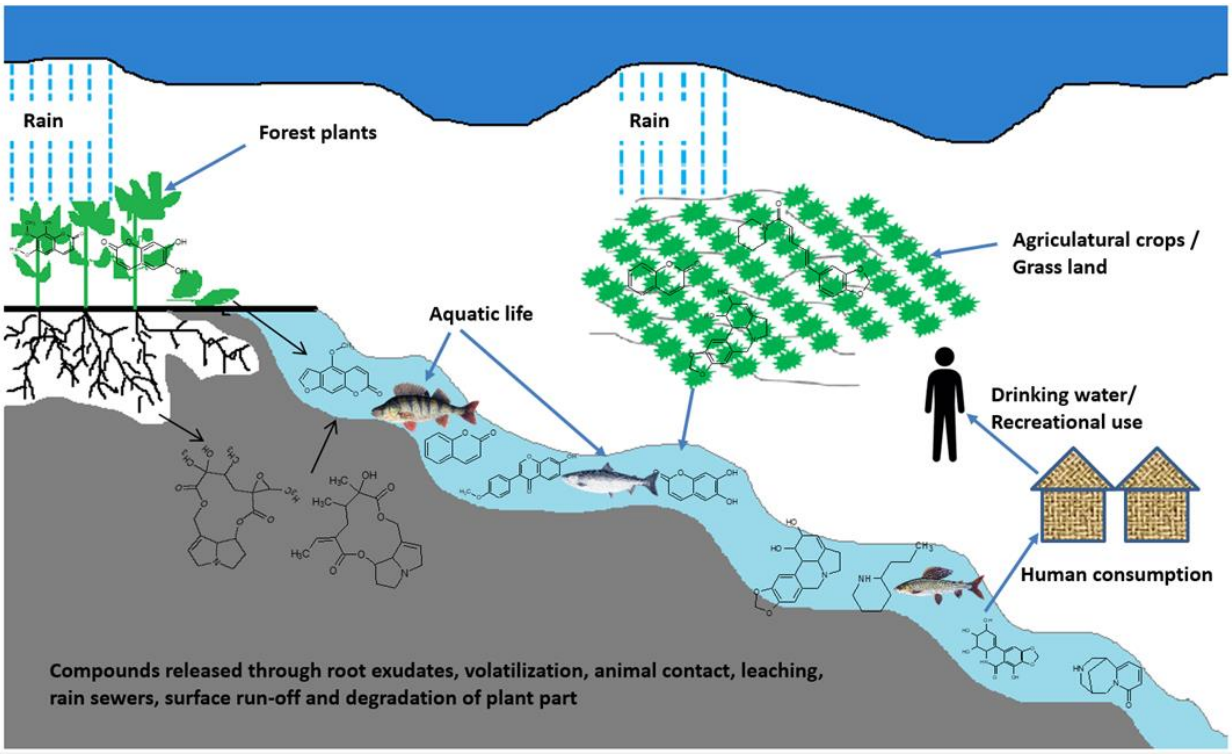

Figure 2: Conceptual depiction of the emission of PSMs to the environment and their route to the river water.

Like other anthropogenic contaminants, PSMs introduced into the environment get distributed among the four major environmental compartments, namely air, water, soil, and biota (living organisms). The fraction of the PSMs that will partition into each compartment is governed by their physicochemical properties (Speight 2017; Schönsee and Bucheli 2020). In addition, the distribution of PSMs in the environment is affected by several phenomena or physicochemical processes such as adsorption, absorption,

dilution, hydrolysis and complexation. Each of these phenomena leads to degradation (by chemical and/or biological processes) or persistence of the compound in the environment (Lofrano et al. 2020; Speight 2017).

The impact of PSMs on the environment is determined by the amount of the chemical that is released, its type and concentration, and where it is found (Bode and Dong 2015; Clauson-Kaas et al. 2014; Gunthardt et al. 2018). Some PSMs can be harmful if released to the environment even when there is no immediate or visible impact (Molyneux et al. 2007; Speight 2017; Hoerger et al. 2009a). Some PSMs are of concern as they may work their way into the living organisms and/or persist in the environment for many years, even for decades (Speight 2017). Once chemicals (i.e. PSMs) are in the environment, it can be very difficult to clean them up or to prevent their migration to areas far from where they were originally introduced (EEA 2018). Thus, the knowledge on the occurrence and source of such compounds in the environment is necessary. However, this is currently hardly available and therefore this thesis is intended to fill this huge gap.

1.6 PSMs of particular concern for water resources

The principle reason for studying the occurrence of PSMs in river waters is not only the search for a better understanding of their effects on the environment (e.g. the aquatic system), but also on human health through unforeseen side effects. So far, in surface water only a subset of diverse group and numbers of PSMs have been discovered in the aquatic environment, mostly at lower concentrations. Despite the occurrence of a small fraction of potentially relevant PSMs at quantities low enough to cause the associated adverse effects, they might pose a risk to organisms due to cumulative effects as a result of contentious exposure (Bucheli 2014).

Gunthardt and colleagues developed a database, Toxic Plants-PhytoToxins (downloadable from https://www.agroscope.admin.ch/agroscope/en/home/publications/apps/tppt.html), containing 1506 toxic phytochemicals of potential ecotoxicological relevance in Central Europe linked to 844 plant species (Gunthardt et al. 2018). The authors characterized the phytotoxins regarding occurrence of plant species as the origin of the compounds; environmental behavior based on aquatic persistence and mobility;

and toxicity. Based on the *in silico* predicted data approximately 41 % (612) of phytotoxins were persistent, mobile and toxic. Thus, showing their potentiality to enter the aquatic environment (e.g. river water) they may cause adverse effect on aquatic organisms. Some of these compounds have already demonstrated their potentiality to aquatic contamination. For instance, Hoerger and colleagues identified the phytoestrogenic isoflavone formononetin in water draining from agricultural land covered with red clover (Hoerger et al. 2009b). Other studies reported the occurrence of hepatotoxic pyrrolizidine alkaloids such as retrorsine and senecionine in surface water impacted by the massive presence of ragwort (*Senecio jacobaea*) at concentration up to several µg/L (Hama and Strobel 2019). Similarly, the mutagenic and carcinogenic ptaquilosides and its degradation products such as ptersion B and ptersion G were detected in surface water draining through abundant presence of Bracken fern (*Pteridium aquilinum* [L.] Kuhn), as potential sources of these compounds (Clauson-Kaas et al. 2014; Clauson-Kaas et al. 2016). However, this is probably only the tip of the iceberg and there is a myriad of PSMs occurring in the environment that might be toxic, and in any case, they add to the complex mixture of anthropogenic and natural compounds in the environment. Since it was impossible to analyse all possible compounds, approximately 160 PSMs were prioritized for target screening by taking the phytotoxins in the database (Gunthardt et al. 2018) as the basic population of candidate compounds. The selection was based on the criteria set by Gunthardt *et al* 2018 and also considering commercial availability and the probability of occurrence due to the abundance of the plants identified as the origin of these metabolites (see Annex A2 and A3).

1.7 Analytical approaches for detection of PSMs in river water

Water analysis has been an important area since the beginning of analytical chemistry. The primary task of water analysis is to provide information on the composition of aqueous samples of diverse origin. This has not really changed since the very beginning of analytical chemistry (Schollée and de Voogt 2012; Marta and Sara 2014). The focus has since shifted substantially from minerals to a multitude of directions; in particular, organic compounds at concentrations down to the sub-nanogram per liter level at present. This was possible only because of numerous innovations in instrumentation in recent decades.

In addition to the high demands on sensitivity, high throughput by automation and short analysis times are major requirements (Schmidt 2018). Water samples can contain complex mixtures of many organic compounds such as PSMs at low concentrations. Thus, as in all measurements, one needs to acknowledge that we will never be able to measure everything in a sample (Schmidt 2018).

PSMs are quite variable in their chemical structures and physicochemical behavior, so there exist a variety of analytical chemical approaches to determine them (van Egmond 2004). Since they occur in surface water and show mainly medium to higher polarity, separation techniques such as liquid chromatography (LC) or gas chromatography (GC) are more convenient approaches to achieve the separation even without derivatization (Picardo et al. 2019). Liquid chromatography (LC) coupled to several detectors such as UV/VIS and fluorescence/chemiluminescence has been implemented, however, the confirmation of the chemical structure of the compound is still in question. Therefore, LC coupled to high resolution mass spectrometry (HRMS) is an ideal technique for the simultaneous identification and detection of PSMs in river waters (Rodriguez et al. 2014; Krauss et al. 2010; Díaz et al. 2012). LC-HRMS is currently the technique of choice because of their excellent sensitivity and specificity, even in samples (e.g. river water) without pre-concentration and clean-up requirements (see Annex A1 and A2).

Chemical screening techniques are widely implemented and the most popular approaches for the detection of contaminants in surface water. According to Krauss et al. (2010), three different analytical approaches can be distinguished depending on the objective of the study: (i) target screening for known compounds – is constrained by the availability of analytical standards and therefore the identification of PSMs or other synthetic contaminants; (ii) suspects screening for non-target compounds – is an ideal approach for the identification of contaminants for which reference standards are lacking, which is the case for a majority of PSMs. It has the advantage of using databases with known analyte structural properties and molecular ion formulae, which are computationally compared to mass spectrometry spectral data to give potential similarities to the compound of interest; (iii) non-target screening for unknown compounds – starts without any prior information on the compounds to be detected (Krauss et al.

2010). Non target screening is a growing focus but more challenging to carry out because it is very difficult to detect and identify trace level contaminants as well as requires extensive data evaluation (Díaz et al. 2012). The environmental samples could contain many thousands of peaks, so that even with the sophisticated instruments and data evaluation workflow, it is not feasible to assign chemical structures for all peaks (Hollender et al. 2017). Since it can be too time-consuming to manually interpret data for thousands of unknown compounds and spectral features, detailed workflows are becoming popular to handle all the data (Richardson and Ternes 2018). As a result, steps need to be taken to decrease the amount of peaks to a manageable number, calculate suitable molecular formulae, determine the isotopic patterns, and perform defect analysis of the mass defect and time prediction of the retention time (Krauss et al. 2010). Depending on the types of samples and availability of resources, several non-target workflows were implemented for the identification of occurrence of contaminants in surface water (Richardson and Ternes 2018; Díaz et al. 2012; Schmidt 2018; Hollender et al. 2017). Although the non-target workflows are often focused on one specific evaluation step, the following key features have emerged: (i) an automated peak detection by exact mass filtering from the chromatographic run; (ii) an assignment of an elemental formula to the exact mass of interest; and (iii) a database search of plausible structures for the determined elemental formula (Krauss et al. 2010). However, in every single step there might be analytes that are excluded totally or partially because of the method used (Schmidt 2018; Schollée and de Voogt 2012). In all the three approaches, the identification of the detected PSMs is based on chromatographic retention time combined with the mass spectrum (Marta and Sara 2014).

1.8 Motivation of the doctoral study

As discussed above, the scientific reports (Clauson-Kaas et al. 2014; Hoerger et al. 2009b; Kolpin et al. 2010; Clauson-Kaas et al. 2016; Gunthardt et al. 2018; Bucheli 2014) provided the motivation to build knowledge on the occurrence of PSMs in the aquatic environments. This is essential and serves as a basis for prioritizing candidates that must be monitored and consequently regulated in terms of emissions and remediation.

The main objective of the present thesis was to identify the occurrence and potential risk of PSMs in surface water impacted by the abundant presence of massive stands of plant species – both natural and agricultural. Secondly, to explore the link between the abundant occurrence of specific vegetation along the river water and detection of their corresponding PSMs in river water. Thirdly, to explore the correlation between rain and leaching of PSMs to the receiving water (river water). And finally, to estimate the possible risk associated with the presence of PSMs using the threshold for toxicological concern (TTC) concept developed for drinking water contaminants.

Chapter Two

Summary Results and Discussion

2.1 Summary results

In this study, the occurrence of PSMs in river waters was investigated by considering three catchments; two of them located in the north-western part of the federal state of Saxony (close to the city of Leipzig) and in Saxony-Anhalt (Bode area), Germany while the third catchment is in Vejle – Haraldskaer area located in the region of southern Denmark. The catchments were selected on the basis of abundant distribution of vegetation along the sampled rivers (see Annex A1-A3 for details on vegetation), which could leave their metabolite fingerprints in the nearby rivers. Several water samples were collected repeatedly from rivers in the catchments over time including before and after rain events.

For the detection of PSMs in river waters, two chemical screening approaches were employed. First, non- target screening (NTS) involving an approach to explain unknown peaks of high intensity in LC-HRMS. Here, NTS with the impact of vegetation on the chemical mixture of river water was demonstrated by detecting numerous overlapping peaks between plant and water – probably stemming from PSMs. Second, target screening involving a large list of known and previously reported PSMs (about 160) prioritized based on their *In Silico* predicted data on their potential toxicity, persistence and mobility as well as their likelihood to occur in the aquatic environment. The experimental setup implemented in this study represents an ideal approach for the detection of such metabolites in river waters, and thus is an important step towards the successful identification and quantification of metabolites. Consequently, the main findings were:

- Vegetation abundantly growing along the surface water potentially impacts the chemical composition of surface water; this was confirmed by investigating rivers draining through areas covered by a large presence of vegetation (Annex A1-A3). Several thousands of overlapping LC-HRMS chemical signals (peaks) could be obtained between river water and plant species from adjacent location. Among the overlapping pairs of peaks (non-target screening, Annex A1), the chemical structure of 12 metabolites were confirmed. On the other hand, several peaks

whose MS/MS and retention time matched but, due to data limitation for structural elucidation their final confirmation were not done. Nevertheless, they represent the same chemical identity suggesting that more PSMs could be identified in river water. Our results demonstrated comprehensively how certain metabolites or metabolite classes correlated to vegetation coverage in the catchment and depend on the season.

- In target screening, a total of 33 PSMs (12 in samples from Germany while 27 from Denmark) belonging to different compound class such as flavonoids, alkaloids, coumarins and other miscellaneous compounds were detected in river waters. The predominant compound classes were flavonoids and alkaloids in rivers from Germany and Denmark, respectively. The toxic alkaloids coniine, lycorine and narccessline and coumarins psoralen and bergapten as well as flavonoids formononetin and daidzein are among the detected compounds.
- In both target and non-target screening, the detected compounds were measured typically in concentrations ranging from ng/L to several µg/L. Higher concentrations as well as the number of metabolites were detected in samples from heavy rain events compared to lighter rain. This clearly showed the impact of rain on the transport of PSMs to the receiving river water.
- Since no sufficient toxicity data is available on the detected PSMs, the threshold for toxicological concern (TTC) concept developed for the non-genotoxic and non-carcinogenic contaminants of drinking water was implemented to make a conservative risk estimate. Consequently, the individual concentrations for several detected PSMs exceeded the TTC value (0.1 µg/L) indicating the occurrence of such compounds would pose a risk to humans, if the water is used for human consumption and recreational purposes.

2.2 Unraveling the impact of vegetation on the chemical composition of surface water using non-target screening

In river waters, using LC-HRMS based target screening, typically large number of chemical signals often with high peak intensity remain unidentified. These chemical signals may represent natural compounds released from plants, animals and

microorganisms, which may contribute to the cumulative toxic risk. In this respect, the present study unraveled the impact of vegetation on the chemical mixture of river water (Annex A1). Here a screening approach involving discriminating non-target peaks occurring between aqueous plant extracts and water samples from adjacent location was used. Eight water samples were collected from two extreme weather conditions – rain event and dry weather from two catchments in Germany – ELP (Elster, Luppe and Pleiße) and Bode catchments (Annex A1). Plant species *Allium ursinum, Galanthus nivalis, Fraxinus excelsior, Digitalis purpurea* and *Conium maculatum L*., found abundantly alonf the rivers were also sampled. For all pairs of water samples (dry weather and rain event), a large number of peaks could be obtained in water samples from rain event compared to dry weather. Analogous to water pairs, the occurrence of a huge number of overlapping peaks could obtain between aqueous plant extracts, from close vicinity to the water sampling spot, and water samples from rain event compared to dry weather samples. This obviously disclosed the impact of rain event on the chemical composition of surface water – whatsoever were the origin of the detected peaks. This finding also supports the hypothesis that rain events drive the leaching of organic compounds (e.g. PSMs) to the river water (see below).

On the attempt made to assign the chemical identity for the overlapping peaks, chemical structures were allocated for 12 compounds, of which nine are PSMs and three are other metabolites (Annex A1). The identified PSMs belongs to different compound classes such as flavonoids (and their glucosides), coumarins and purine nucleobases – flavonoids being the predominant class. In general, most of the identified metabolites contain one or more phenolic groups representing a class of compounds found most abundantly in vegetation (Puri et al. 1998). Meanwhile the remaining overlapping pairs of peaks contained several pairs of peaks whose MS/MS spectra match. Nevertheless, no chemical structure suggestions were forwarded using the implemented chemical and spectral databases as the non-target identification tool. However, the pairs of peaks with matching MS/MS spectra represents the same chemical structure. The occurrence of these unidentified peaks added on the confirmed peaks attests the impact of vegetation growing along rivers on the chemical composition of river water. The identified metabolites were obtained in individual water samples at concentrations up to 5 µg/L.

This study also disclosed the influence of rain on the occurrence as well as concentration of PSMs in river water. The concentration and number of individual detected PSMs vary in water samples from the same spot at different rain event days – obviously at different rain intensity. This could be attributed to the difference in rain intensity during the sampling campaign, which was supported by detecting none of the PSMs in samples from dry weather (Annex A1). Vegetation coverage could also affect the concentration of metabolites, this was demonstrated by obtaining individual concentrations of up to 5 µg/L (apiin) in water samples collected during less intense rain but with massive presence of *Digitalis purpurea*. A similar result was reported by Clauson-Kaas and colleagues who conducted an investigation on monitoring the level of ptaquiloside in the stream draining a bracken-infested catchment base flow and in response to rain event during a growth season (Clauson-Kaas et al. 2016). The authors reported the substantial difference in the concentration of ptaquiloside in two types of samples, which were measured up to 61 ng/L and 2 µg/L in base flow and rain event samples respectively. The rain event concentration was reproducible in the time lag of approximately 1 h from onset of rain to elevated concentrations, and returning rather quickly (about 2 h) to base flow concentration levels. This clearly showed the influence of rain events on the transport of PSMs from the synthesizing plant to the receiving surface water as well as the temporal connection between rainfall and concentration of PSMs (Clauson-Kaas et al. 2016). A study by Hama and colleague also disclosed the importance of rain events as the main driving factor for transporting alkaloids from plants to soil and water (Hama and Strobel 2020). In fact, there are several other determining factors such as the soil pH, topography, hydrology, and vegetation coverage, which will evidently affect the level of PSMs in the receiving stream, as well as the distance from vegetation (Clauson-Kaas et al. 2016).

2.3 Occurrence of PSMs in river waters using target screening

After unraveling the impact of specific vegetation on the chemical mixture of river water (by non-target screening), target screening aimed at detecting more PSM fingerprints in river water using pre-selected target compounds (about 160 PSMs) was performed. The targets were selected with the help of *In silico* predicted data with a high probability to reach the aqueous environment due to mobility and persistence as well as the abundance

of the plants as the origin of these compounds (Annex A2 and A3). To achieve our objective, water samples were collected from two European countries. From Germany 38 rain event grab water samples, for direct injection (DI) to LC-HRMS, complemented with 15 dry weather samples (as control) and from Denmark 20 large volume solid phase extraction (LVSPE) rain event samples were collected. During sampling, the surrounding environment for the sampled rivers in Germany were mainly dominated by monocotyledonous and tuberous plants such as *Allium ursinum*, *Anemone nemorosa*, *Galanthus nivalis* and *Leucojum vernum* as well as other forest trees such as *Quercus robur*, *Fraxinus excelsior*, *Acer pseudoplatanus* and *Ulmus*. Upstream to the sampling spots of some rivers, there were also agricultural fields covered with crops such as winter wheat, triticale, winter barley, rye, rape, sugar beet and corn. Similarly, in close vicinity to the sampling sites in Denmark, the rivers drain through agricultural land with barley, wheat and sugarbeet, forest with high abundance of *Alnus glutinosa* (common alder), *Petasites hybridus* (butterbur), *Symphytum officinale*, *Urtica dioica* and grassland with *Senecio Jacobaea* L. The screening results revealed the occurrence of a total of 33 target compounds in samples from both countries, of which 6 were common to both countries (Figure 3). Among the 33 detected targets, 12 were obtained in about 50% of rain event samples from Germany and 27 targets were observed in all samples from Denmark. The target compounds were detected in none of the dry weather samples. The detected target compounds belong to different compound classes such as alkaloids, coumarins, flavonoids and other miscellaneous compounds. In general, many of the identified metabolites contain one or more phenolic groups representing a class of compounds found most abundantly in vegetation. In both countries, the simple coumarins fraxidin and scopoletin were obtained at higher frequency compared with other compounds (Figure 3). Out of 27 detected compounds in Denmark, about 50% of these were detected in over 80% of the samples. The targets detected in common were the simple coumarins fraxidin, scopoletin and aesculetin and the furanocoumarin psoralen as well as an alkaloid piperine, a compound most widely used as spices worldwide (Figure 3). The simple coumarins are synthesized by several plant species in the environment (Afendi et al. 2012), which could be the cause for their ubiquitous occurrence in both countries. The rest of the target compounds are mainly plant specific, and thus, could be released from

specific plant species (as a potential source of these target compounds) available along the sampled rivers. Since the catchments from both countries have different vegetation, the distribution pattern of detected target compounds also varied. For instance, the occurrence of the pyrrolizidine alkaloids such as intermedine and echimidine in Denmark were correlated to the massive presence of *Symphytum officinale* along the rivers. The same is true for lycorine and narciclasine in Germany, which could be linked to the dominant plant species from Amaryllidaceae family (e.g. *Galanthus nivalis* (snow drop), *Leucojum vernum* and *Anemone nemorosa*). Although some plants such as *Senecio Jacobaea* L found predominantly, its typical known pyrrolizidine alkaloids such as senecionine, jacobine, erucifoline and seneciphylline were not detected in any of the rivers from adjacent location. However, these and other pyrrolizidine alkaloid compounds were reported previously in surface water at the concentration range of 4-270 µg/L, which was impacted by the massive standards of *Senecio Jacobaea* L (Hama and Strobel 2019).

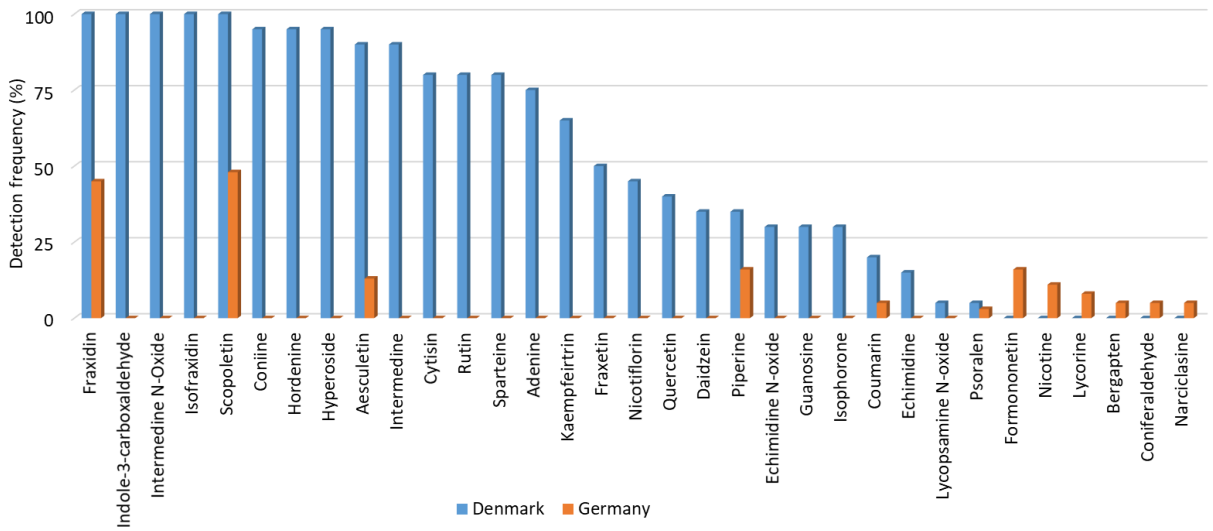

Figure 3: Detection frequency of target compounds identified in water samples from Germany (38 DI rain event samples) and Denmark (20 LVSPE samples).

Some compounds such as piperine, isophorone and nicotine are naturally occurring compounds synthesized by plants, however their presence in river water could be directly linked to human activities. These compounds were detected in rivers influenced by anthropogenic activities in rivers flowing through industrial or urban areas. The

compounds were not detected in any of the rivers with limited human impact or from pristine areas. This could also signify that the compounds were introduced through wastewater or other human related activities. Some flavonoid compounds such as quercetin, keampferol and apiin are most abundant in edible plants (vegetables and fruits), thus there could also be a possibility for these compounds to be released though municipal waste or landfill leachates. Moreover, due to lack of appropriate monitoring techniques for the exclusion of such compounds from effluent of waste water treatment plants, they could escape the treatment to the receiving river water.

2.4 Distribution of detected PSMs in river waters (target and non-target screening)

In this study, by target and non-target screening, a total of 38 PSMs in rivers from both Germany and Denmark were detected (Table 2), of which 63% were measured in rivers from Germany while 71% in Denmark. Among the detected PSMs from both countries, 38% (14) were alkaloids, 30% (11) flavonoids, 23% coumarins and the rest were other miscellaneous metabolites. In Germany, flavonoids were the predominant compound classes detected while alkaloids in Denmark. This might be correlated to the difference in the distribution of vegetation in the sampling environment (Annex A1-A3). The compounds were obtained in concentrations ranging from units of ng/L to several thousands of ng/L. Relatively higher concentrations of target compounds were measured in samples from Germany.

Table 2: Phytochemicals identified in river water samples from Germany and Denmark using both non-target and target analysis.

Compound class	Phytochemical	Formula	CAS	m/z	RT (min)	Concentration (min - max, ng/L)	
						Germany	Denmark
Alkaloids	Coniine	$C_8H_{17}N$	458-88-8	128.1433	7.1	ND	3.3 - 400.5
	Cytisin	$C_{11}H_{14}N_2O$	485-35-8	191.1179	0.5	ND	3.8 - 24.8
	Echimidine	$C_{20}H_{31}NO_7$	520-68-3	398.217	6.4	ND	3.4 - 4.2
	Echimidine N-oxide	$C_{20}H_{31}NO_8$	41093-89-4	414.2117	6.5	ND	13.5 - 34.7
	Hordenine	$C_{10}H_{15}NO$	539-15-1	166.1226	0.5	ND	5 - 21.9
	Indole-3-carboxaldehyde	C_9H_7NO	487-89-8	146.0601	6.8	ND	5.3-108.5
	Intermedine	$C_{15}H_{25}NO_5$	10285-06-0	300.1801	0.7	ND	1.2 - 12.5
	Intermedine N-oxide	$C_{15}H_{25}NO_6$	95462-14-9	316.1752	0.8	ND	4.2 - 47
	Lycopsamine N-oxide	$C_{15}H_{25}NO_6$	95462-15-0	316.1751	0.5	ND	3.2
	Lycorine	$C_{16}H_{17}NO_4$	476-28-8	288.1225	1	1015 - 2331	ND
	Narciclasine	$C_{14}H_{13}NO_7$	29477-83-6	308.0765	5.7	507 - 3353	ND
	Nicotine	$C_{10}H_{14}N_2$	54-11-5	163.1228	0.9	2 - 35	ND
	Piperine	$C_{17}H_{19}NO_3$	94-62-2	286.1434	12	1 - 338	0.3 - 18.1
	Sparteine	$C_{15}H_{26}N_2$	90-39-1	235.2168	0.7	ND	4.4 – 10.8
Flavonoids	Alpinetin	$C_{16}H_{14}O_4$	36052-37-6	271.0962	10.3	23 - 500	ND
	Apiin	$C_{26}H_{28}O_{14}$	26544-34-3	565.1547	9.1	1200 - 5100	ND
	Cynaroside	$C_{21}H_{20}O_{11}$	1268798	449.1073	8.6	200 - 2100	ND
	Daidzein	$C_{15}H_{10}O_4$	486-66-8	255.065	9.5	ND	84.7 - 281.9
	Formononetin	$C_{16}H_{12}O_4$	485-72-3	269.0804	10.8	8 - 123	ND
	Hyperoside	$C_{21}H_{20}O_{12}$	482-36-0	465.1017	8.7	3800 - 4000	3.9 - 51.6
	Kaempferitrin	$C_{27}H_{30}O_{14}$	482-38-2	579.1707	9.3	900	5.5 - 51.8
	Kaempferol	$C_{15}H_{10}O_6$	520-18-3	287.0548	10.6	NQ	ND
	Nicotiflorin	$C_{27}H_{30}O_{15}$	17650-84-9	595.165	9.3	1900 - 2200	5.2 - 15.9
	Quercetin	$C_{15}H_{10}O_7$	117-39-5	303.0496	8.6	1900 - 2500	11.3 - 36.5
	Rutin	$C_{27}H_{30}O_{16}$	153-18-4	611.1604	8.7	ND	5.2 - 191
	Trifolin	$C_{21}H_{20}O_{11}$	23627-87-4	449.1073	9.1	300 - 2900	ND
Coumarins	Aesculetin	$C_9H_6O_4$	305-01-1	179.0336	3.6	104 - 1658	7 - 34.8
	Bergapten	$C_{12}H_8O_4$	484-20-8	217.0495	10.1	510 - 541	ND
	Coumarin	$C_9H_6O_2$	91-64-5	147.0441	7.3	12 - 43	4.5 - 9.5
	Fraxetin	$C_{10}H_8O_5$	574-84-5	209.0443	6.2	ND	10 - 29.3
	Fraxidin	$C_{11}H_{10}O_5$	525-21-3	223.06	7.8	19 - 1145	4.9 - 16.3
	Isofraxidin	$C_{11}H_{10}O_5$	486-21-5	223.0599	7.4	20 - 300	5.9 - 49.1
	Psoralen	$C_{11}H_6O_3$	66-97-7	187.0388	9.2	141 - 224	5
	Scopoletin	$C1_0H_8O_4$	92-61-5	193.0496	7.1	7 - 49	5.5 - 22.6
Other miscellaneous compounds	Adenine	$C_5H_5N_5$	73-24-5	136.0619	0.5	400 - 2600	0.5 - 6.1
	Coniferyl aldehyde	$C_{10}H_{10}O_3$	458-36-6	179.0701	7.6	13 - 46	ND
	Guanosine	$C_{10}H_{13}N_5O_5$	118-00-3	284.0984	1	1100 - 4000	2.4 - 5.8
	Isophorone	$C_9H_{14}O$	78-59-1	139.1117	9	ND	12.2 - 25.1

2.4.1 Alkaloids

Alkaloids were obtained in both countries and more widely detected than other compound classes. Among the identified 38 phytochemicals, 14 were from a compound class alkaloid which includes the sub class such as pyrrolizidine, quinolizidine, indole, piperidine, indolizidine and polyketide-derived alkaloids. The pyrrolizidine alkaloids intermedine, echimidine and their N-oxides such as lycopsamine N-oxide were detected at concentration range of 1.2-47 ng/L in Denmark. The toxicity study on rats using lycopsamine and intermedine from extracts of *Symphytum officinale* demonstrated adverse effects such as angiectasis at 1500 mg/kg (LD_{50} for single intraperitoneal injection in rats) (Brauchli et al. 1982). The same study reported similar effects on chick liver at concentrations of approximately 77 mg/kg (Brown et al. 2016). Although pyrrolizidine alkaloids occur at levels that are too low, as detected in the present study, to produce acute liver damage, they are high enough to be of concern as a possible long-term cause of cirrhosis and liver failure (Wiedenfeld and Edgar 2011; Aniszewski 2007; van Egmond 2004). The toxic alkaloids coniine, lycorine and narciclasine were detected in Germany at concentrations of 0.4, 2.3 and 3.4 µg/L – the highest among detected alkaloids. Besides their bioactive properties (see introduction), their toxic effects on living organisms were reported. For instance, lycorine demonstrated acetylcholinesterase inhibition effects at LC_{50} of 213 µg/L while coniine acts as a nicotinic acetylcholine receptor antagonist, which can lead to inhibition of the nervous system, eventually causing death (López et al. 1999; Hotti and Rischer 2017; Hotti et al. 2015). The neurotoxic alkaloid coniine from poison hemlock was also frequently detected in Denmark. Perhaps, coniine's most famous victim is Socrates who was sentenced to death by poison chalice containing poison hemlock in 399 BC (Hotti and Rischer 2017).

The quinolozidine alkaloids cytisin and sparteine, main constituent of Lupinus and other plant species from Fabaceae family, were among the frequently detected targets in Denmark, only. Recently, Hama and Strobel reported the occurrence of both alkaloids in soil pore water collected from agricultural land area covered by *Lupinus* species (Hama and Strobel 2020). The authors reported the link between the occurrence of alkaloids in water with the presence of Lupine and precipitation as a driver of the compounds to the

water body. They also stated that higher concentration alkaloids were registered during rain events, while these were comparatively lower in non-rain event samples (Hama and Strobel 2020). This is in agreement with the present study in which higher concentration and number of metabolites per sample correlated with rain intensity.

In general our result disclosed the occurrence of natural alkaloids in rivers from the area impact by large presence of vegetation along the rivers. The identified alkaloids belonged to different sub classes such as pyrrolizidine alkaloid, quanolizidine, piperidine and indole alkaloid – of which PAs occur predominantly. Alkaloids stemming from human activities such as piperine and nicotine, once consumed by population and collected in sewer lines, tend to flow to municipal waste water treatment plants which are usually not designed to remove them. Some toxicity data are available, but further studies on the exposure and toxicity of many alkaloids are required to draft suitable risk assessment strategies (Günthardt et al. 2020).

2.4.2 Flavonoids

Several natural flavonoids were detected in both countries – prominently in Germany. Their overall concentrations ranged from 3.9 to 5100 ng/L. Flavonoids are the most abundant compounds in plant species specifically in edible plants by humans such as fruits and vegetables. Thus, in addition to the growing vegetation, their emission to river waters could be linked to the municipal solid waste (MWS) were the landfill leachate is a potential source of these compounds. MSW contain everyday by-products such as food wastes. The composition of waste present in landfills coupled with the quantity of leachate generated due to rain will determine the type and concentration of chemicals emitted to the receiving water from the landfill leachate (Lofrano et al. 2020). For instance, the isoflavone daidzein, a naturally occurring isoflavone exclusively found in soybeans and other leguminous plants widely used as a source of food for humans, was detected only in Denmark. Thus, its occurrence in surface water is more strongly linked with municipal waste since such plants are not cultivated in Denmark. This was evidenced by absence in rivers impacted by massive presence of forest, other agricultural crops and grasses. Instead, detected in rivers with several tributaries (along its way from origin), from which it might get loaded with daidzein. The concentration of daidzein increases with rain

intensity, which could be attributed to its rain facilitated emission through washing from landfill leachates. Daidzein was previously reported at a maximum concentration of 5.5 ng/L in surface water from Switzerland impacted by agricultural vegetation (Günthardt et al. 2020). Another isoflavone of similar structure, formononetin was also detected at concentration up to 123 ng/L but only in Germany. This compound was previously reported as most frequently occurring compound in surface water impacted by natural (Günthardt et al. 2020) and agricultural (Hoerger et al. 2009b) vegetation from Switzerland. It was also reported in surface water impacted by large presence of agricultural crops in the USA (Kolpin et al. 2010). In the present study, formononetin was measured at higher concentration than in the USA (13.5 ng/L) and at lower concertation (217 ng/L) than in Swiss surface water (Kolpin et al. 2010; Hoerger et al. 2009b).

The glucosides of flavones apigenin – apiin and luteolin – cynaroside were among the identified naturally occurring flavonoids in Germany. Their occurrence in river water is linked to *Digitalis purpurea*, a plant abundant along the sampled river (upstream to the sampling spot) (Annex A1). Cynaroside was also detected in Amaryllidaceae such as *Allium ursanium* and *Galanthus nivalis*, specific plants from a specific season, which show a high abundance within short growth periods in early spring. Apiin was detected in river water at higher concentration up to 5 µg/L.

The occurrence of a flavonol kaempferol and its glucoside nicotiflorin, trifolin and kaempferitrin were identified in both countries with the exception of kaempferol and trifolin in Denmark. These compounds are widely distributed in several vegetation – both natural and agricultural (Afendi et al. 2012). However, in the present work, their occurrence in river water is linked to several specific plant species – for instance, kaempferol from *Galanthus nivalis,* nicotifilorin from *Fraxinus exclsivor* and trifolin from both plants as well as from *Allium ursinium*. These plant species found most abundantly along the studied rivers, which could explain the likely discovery of these compounds in the rivers. Another flavonol quercetin and its glucosides rutin and hyperoside were also detected in surface waters – hyperoside in both countries while rutin and quercetin in Denmark and Germany, respectively. Similar to other flavonols, they (quercetin and hyperoside) were introduced by *Galanthus nivalis* and *Fraxinus exclsivor*.

This study demonstrated the emission of flavonoid compounds from natural vegetation (e.g. forest plants) and agricultural crops. The emission could also be done through municipal wastes. Once these compounds enter the environment, they experience the same fate as anthropogenic compounds. Thus, a monitoring study is required in order to mitigate unknown and unforeseen adverse effects, which might be caused by the PSMs. The presence of such compounds in addition to the existing anthropogenic contaminants could enhance the toxic effect on the aquatic organisms. Thus, making the surface water even worse for the aquatic organisms. Numerous preclinical studies have shown that the flavonoids and some of their glycosides have a wide range of pharmacological activities (see introduction) (Calderón-Montaño et al. 2011; Wang et al. 2018). Additionally, their toxic effects due to continuous exposure were also reported, for instance, the flavonols quercetin and kaempferol were associated inversely with lung cancer among tobacco smokers, but not among nonsmokers (Cui et al. 2008).

2.4.3 Coumarins

Unlike synthetic coumarins, naturally occurring coumarins have not been intensively studied in surface waters. At the same time, these compounds are known constituents of dissolved organic matter. A previous study analyzed aqueous solutions of isolated organic matter that reflects the presence of simple coumarins in the corresponding natural water samples (Remucal et al. 2012). In this study, natural coumarins of plant origin were detected in river waters. The ubiquitous occurrence of simple coumarins, regardless of the type of surrounding vegetation, in rivers from both Germany and Denmark corroborates their likely synthesis route via a variety of plants. Classes of coumarin, furanocoumarins such as psoralen and bergapten (5-methoxypsoralen) were detected up to a maximum concentration of 224 and 541 ng/L, respectively. Both compounds synthesized by the plant species from Apiaceae, Fabaceae, Moraceae and Rutaceae family (Afendi et al. 2012). Various citrus species such as lime and orange from the family of Rutaceae contain significant amounts of furanocoumarins bergapten and psoralen (Dugrand-Judek et al. 2015; Melough et al. 2018). The citreous species are extensively consumed by human thus, besides their emission from vegetation, they could also reach river water though municipal waste. The detected simple coumarins and furanocoumarins

demonstrated several biological properties (see introduction). They also displayed toxic effects, for instance, furanocoumarins are potent photosensitizers when activated by near-UV light (300 – 380 nm), thus they are phototoxic, mutagenic and photocarcinogenic (Schlatter et al. 1991; Walter et al. 1982). Severe dermatitis can result after contact with furanocoumarin containing plants in the presence of sunlight. Epidemiological data suggested that relatively high levels of dietary exposure to furanocoumarins may also increase risk of skin cancer (Ceska et al. 1986; Melough et al. 2018). Thus, their presence in surface water needs consideration if the water is used for drinking water abstraction and recreational purposes or serves as habitat for aquatic life.

2.4.4 Other miscellaneous compounds

This study also unraveled the occurrence of other classes of compounds such as purine nucleosides, cyclic ketone and aldehyde containing phenolic functional group. The purine nucleosides, adenine and guanosine were detected in rivers but both compounds are not solely plant-produced compounds (rather generic from any biota). However, in this study, their occurrence in river is attributed to plants such as *Allium ursanium, Galanthus nivalis, Fraxinus exclsivor, Digitalis purpurea* and *Conium maculatum L*. Lignin components of a plants coniferyl aldehyde and a cyclic ketone isophorone were detected in Germany and Denmark, respectively. They could be synthesized by several vegetation – coniferyl aldehyde from Asteraceae family (e.g. Petasites tricholobus) and Fagaceae (e.g. Quercus spp.) (Afendi et al. 2012; Harborne and Baxter 1999) and isophorone from *Brassica hirta* (Miyazawa and Kawata 2006) and *Prunus armeniaca* L (Gomez et al. 1993). There is no evidence for the presence of such plants along the rivers, where the compounds were detected. However, the occurrence of isophorone is very likely due to anthropogenic activity, since it is widely used as solvent in industries (USA-ATSDR 2018).

2.5 Risk estimation of PSMs in river water

Due to the lack of sufficiently available data on the PSMs regarding toxicity, the threshold for toxicological concern (TTC) concept can be one way to make a conservative estimate of the potential risk. Accordingly, in this study, the TTC value of 0.1 µg/L (Mons et al. 2013) suggested for all organic chemicals which are not genotoxic and no steroid

endocrine disruptors for drinking water was used. And also, since there is no TTC values available for untreated (surface) water, the one for drinking water was adapted for risk estimation. Consequently, most of the detected compounds, in this study, exceeded the TTC value, thus caution should be taken while using such surface waters for drinking water abstraction or recreational use.

The individual TTCs do not consider mixture effects, thus additive risk quotients (RQs) in agreement with typical risk assessment approaches normalizing concentrations to the effect or threshold concentrations were determined. Adding RQs is in agreement with the concept of concentration addition for mixture risks although using TTC as proxies for effect concentrations is questionable. However, as a conservative estimate for mixtures of organic chemicals with unknown effect concentrations (e.g. PSMs) it might be appropriate (at least alternatives are lacking). In all the samples the detected compounds occur as a mixture of at least 3 compounds per individual sample, even 20 compounds per sample were detected. In most cases, the calculated mixture RQs were found to be greater than 0.1 indicating the occurrence of PSMs in mixture could possibly pose risk to both aquatic life and humans, if the water is used for human consumption and recreational purpose. Thus, the toxic risks by individual PSMs and mixtures thereof and a contribution to overall toxicity of surface waters cannot be excluded and demand for additional efforts in hazard characterization.

The individual compounds are observed in concentrations that are generally considered too low to cause acute effects. Nevertheless, health effects due to long-term exposure to a mixture of low concentrations of all kinds of emerging contaminants (e.g. PSMs) cannot be excluded with current knowledge (Houtman 2010).

Chapter Three

Conclusion and Future Outlook

3.1 Conclusion

In this thesis the occurrence of PSMs in river waters were investigated using LC-HRMS based non-target and target screening. The studies (Annex A1-A3) revealed the occurrence of several PSMs belonging to the compound classes such as alkaloids, coumarins and flavonoids, some of them occurring frequently. In many cases, concentrations of these compounds, which are known to exhibit substantial biological activity, exceeded the concentrations of many anthropogenic chemicals in surface waters. For instance, the toxic alkaloids lycorine and narciclasine were detected at concentrations of 2.3 and 3.4 µg/L, respectively, even 5 µg/L were obtained for Apiin. Thus, the concentration levels of PSMs found in rivers, can possibly cause adverse effects on aquatic life and humans. Although this finding does not necessarily indicate toxic risk to aquatic organisms, it may illustrate the relatively high concentrations at which a contribution to mixture toxicity cannot be excluded. So far, unlike anthropogenic triggered compounds, naturally occurring compounds are not included in environmental monitoring programs due to limited knowledge on their occurrence and effects in the environment. Thus, the present study contributed to minimize this knowledge gap and the identified compounds represent only the tip of the iceberg of possibly toxic PSMs in water resources. Thus, it is recommended to consider PSMs in monitoring and risk assessment and also in the context of drinking water abstraction. A potential risk particularly during rain events promoting the leaching of PSMs to surface waters and massive occurrence of toxic plants in specific seasons may not be excluded. Due to lack of aquatic toxicity data for PSMs and extremely scarce exposure data, no reliable risk assessment and prioritization of PSMs for monitoring and assessment can be performed. Thus, PSMs should be included increasingly into chemical monitoring of surface waters to collect exposure data on a larger scale complemented with toxicity testing of compounds occurring frequently or in high concentrations.

3.2 Future outlook

- The PSMs identified in this thesis are the tip of the iceberg of a diverse and wide number of naturally occurring compounds. Thus, extensive chemical screening or identification should be assessed to disclose the occurrence of more PSMs in surface water.
- For the identified compounds in this study, the environmental and ecotoxicity data are rather limited or very few measured data are available and preliminary assessments were only provided by few individual case studies for single compounds. In addition, chemical mixture and potential long-term exposure effects on non-target organisms are unknown. Since the ecotoxicological effect data is needed to assess the risk of PSMs, studies on the exposure as well as toxicity of PSMs detected in surface water are required.
- In order to fully evaluate the ecotoxicological effects of aquatic contaminants, the whole chemical composition, both from anthropogenic and natural origin should be characterized. This will contribute greatly to obtain a holistic picture of water contaminants. Further PSM identification, in addition to anthropogenic contaminants, should also be assessed.
- Unlike anthropogenic contaminants, PSMs are not included in monitoring studies. Thus, apart from their release from vegetation, they could also be emitted through municipal waste or escape through waste water treatment plant. Therefore, PSMs are need to be included in the monitoring studies in order to mitigate their emission through waste water.
- Since PSMs may occur in any surface water impacted by the abundant presence of vegetation, in addition to synthetic contaminants, the use of such water resources for drinking water abstraction should be done in caution.

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Annex: Publications and manuscripts as part of the thesis

A.1 Non-target screening for detecting the occurrence of plant metabolites in river waters

Nanusha MY, Krauss M & Brack W., 2020. Environ Sci Eur (2020) 32:130

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Author's contribution statement

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Contributing authors: Nanusha MY (1), Krauss M (2) and Brack W (3)

What are the contributions of the doctoral candidate and his co-authors?

1) **Concept and design**
   - ✓ Doctoral candidate: 70 %
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Non-target screening for detecting the occurrence of plant metabolites in river waters

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Abstract

Background: In surface waters, using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS), typically large numbers of chemical signals often with high peak intensity remain unidentified. These chemical signals may represent natural compounds released from plants, animals and microorganisms, which may contribute to the cumulative toxic risk. Thus, attempts were made to identify natural compounds in significant concentrations in surface waters by identifying overlapping LC-HRMS peaks between extracts of plants abundant in the catchment and river waters using a non-target screening (NTS) work flow.

Results: The result revealed the presence of several thousands of overlapping peaks between water—and plants from local vegetation. Taking this overlap as a basis, 12 SPMs from different compound classes were identified to occur in river waters with flavonoids as a dominant group. The concentrations of the identified compounds ranged from 0.02 to 5 µg/L with apiin, hyperoside and guanosine with highest concentrations. Most of the identified compounds exceeded the threshold for toxicological concern (TTC) (0.1 µg/L) for non-genotoxic and non-endocrine disrupting chemicals in drinking water often by more than one order of magnitude.

Conclusion: Our results revealed the contribution of chemicals eluted from the vegetation in the catchment to the chemical load in surface waters and help to reduce the number of unknowns among NTS high-intensity peaks detected in rivers. Since secondary plant metabolites (SPMs) are often produced for defence against other organisms and since concentrations ranges are clearly above TTC a contribution to toxic risks on aquatic organisms and impacts on drinking water safety cannot be excluded. This demands for including these compounds into monitoring and assessment of water quality.

Keywords: Natural toxins, Surface water, Emerging contaminant, Phytochemical, Phytotoxin

Background

Surface waters may contain a large number of chemicals detectable as signals in LC-HRMS including a large fraction of unknown chemicals often with high peak intensity [57]. In addition to synthetic chemicals and transformation products thereof, these signals may represent also natural compounds released from plants, animals and microorganisms, which may be not only considered as a confounding factor in chemical and effect-based screening of water contaminants but may also contribute to the cumulative toxic risk of water contamination [18]. Thus, reducing the number of unknowns in water samples by identifying also natural compounds in significant concentrations in surface waters will help to improve monitoring and assessment of water quality potentially impacted by complex mixtures of natural and synthetic compounds as shown recently for carbolines and aromatic amines [46].

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Plants are known to produce a large number of SPMs depending on the species, season and environmental conditions [5, 28, 45]. SPMs play a significant role in controlling essential functions of growth and reproduction [41] and enable the synthesizing plants to overcome temporary or continuous threats and to establish biological and ecological relationships with other organism [5, 41]. SPMs are typically advantageous to the producing plants but may cause adverse effects in other organisms exposed to these SPMs [41]. A wide variety of metabolites are released from plants through microbial decomposition and enzymatic degradation of plant parts with metabolites leaching to the receiving surface water through rain sewers or surface runoff [3, 45]. SPMs are also released by root exudates and volatilization from living plants [45, 49]. SPMs are diverse in their structure and effect on human health and wildlife [1]. Effects of SPMs on human health may be ambiguous. For instance, flavonoids of plant origin are often considered as safe and widely accepted as health promoting phytochemicals. However, experimental in vivo and in vitro studies have produced conflicting results. Some flavonoids (e.g., quercetin, rutin) interact with DNA and/or exhibit carcinogenic activity in rodents shown in male rat for a dose of about 60 mg/kg in vivo [24]. Others have mutagenic (e.g., quercetin) and/or pro-oxidant effects and may interfere with essential biochemical pathways [16, 36]. Isoflavones such as genistein exhibit estrogenic activities [22]. The flavonoids, kaempferol and apigenin act through estrogen-receptor mediated mechanisms and exhibit anti-estrogenic effects at a concentration of 34 and 32 µg/L in in vitro [56]. Several flavonoids including kaempferol and quercetin inhibit cholinesterases (AChE, BChE), with quercetin being most active at IC_{50} of 62 mg/L [7, 23].

Recent studies revealed a large diversity of phytochemicals from different classes of compounds (e.g., formononetin, gramine and senecionine) in environmental samples such as water and soil [18, 20, 47] and concentrations exceeding thresholds of toxicological concern for drinking water (TTC) [42] in water bodies from natural and agricultural areas. Assuming that only a minor fraction of SPMs in surface waters is known, considering these compounds in water quality monitoring and assessment may not rely on target screening only [9, 11, 18, 20, 47]. Here, non-target screening is helpful to access also unknown and unnoticed contaminants such as SPMs using liquid chromatography coupled to high-resolution mass spectrometry LC-HRMS) detecting as many contaminants as possible in parallel. Significant chemical information (e.g. elemental composition, chemical formula, isotopic pattern) can be extracted in a single experiment [33, 34] to be used as input for structure elucidation. In the present study we tested non-target screening as a tool to identify SPMs from surrounding vegetation in water samples using LC-HRMS. The impact of vegetation on the chemical mixture in river waters was investigated by comparing NTS data of water samples with NTS data of eluates of vegetation abundantly present along the examined rivers. Since toxicity data of SPMs are extensively lacking, preliminary toxic risk estimates of individual compounds and mixtures were based on TTCs [42] for environmental contaminants in drinking water being aware that this approach is not directly applicable for surface waters without human consumption. Mixture risks were estimated using the concentration addition model for mixture effects.

Materials and methods

Description of water and plant sampling locations

Study areas are located at the north-western part of the federal state of Saxony (Leipzig floodplain forest along the rivers Elster, Pleiße and Luppe called EPL catchment) and Saxony-Anhalt (Bode catchment), Germany. The floodplain is characterized by the trees *Quercus robur*, *Fraxinus excelsior* and *Acer pseudoplatanus*, while in spring, the forest scrub is dominated by plants from the amaryllis family (Amaryllidaceae) such as *Allium ursinum* and *Galanthus nivalis* [32]. The Bode catchment is characterized by a large diversity of natural and agricultural vegetation along a number of small streams. From both locations, streams with river banks covered by few highly abundant plants of interest were designated for this study. The study in the ELP catchment focused on three small streams and two seasonal plants, *Allium ursinum* and *Galanthus nivalis,* while in the Bode catchment three rivers, namely, Getel, Drängetalwasser and Barrenbach with their corresponding plant species *Fraxinus excelsior*, *Digitalis purpurea* and *Conium maculatum* L., respectively, were selected.

Both plant and river water samples were collected during plant growth season following rain events in the 2019 summer season, based on the hypothesis that under these conditions plants are particularly prone to leave their SPM fingerprints in the aquatic environment [6, 8]. To this end, we collected a total of 8 water samples (see Additional file 1: Table S1) including 5 samples from 3 streams from the ELP catchment and 3 samples from 3 streams in Bode catchment (Fig. 1). Water samples were taken with glass beaker (500 mL) and solids were allowed to settle for about 2 min before transferring to a pre-cleaned glass bottle. Aliquots of 1 mL were transferred to 2-mL autosampler vials for the chemical analysis. Backup samples were frozen in 125 mL Nalgene bottles. The sampling bottles were pre-cleaned and oven dried before use. Bottles were rinsed with the river water prior to sample collection. As a control, five river water samples were

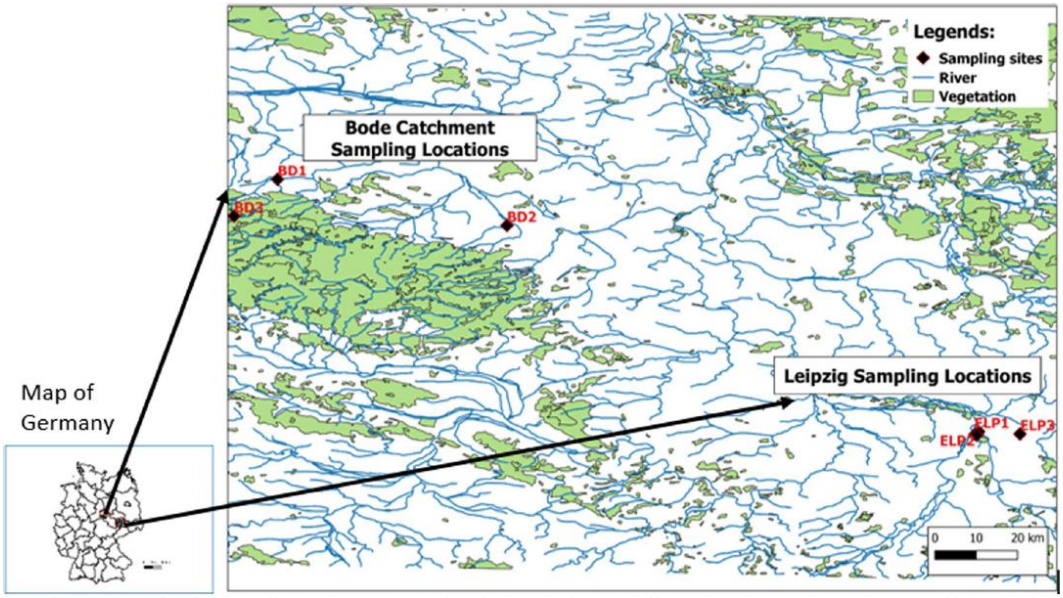

Fig. 1 Map showing sampling locations from Leipzig (ELP) and Bode (BD) catchment. (Abbreviations: ELP1, ELP2, ELP3 are small streams sharing the same vegetation in the Leipzig floodplain forest and BD1 (Barrenbach), BD2 (Getel), BD3 (Drängetalwasser) are streams from the Bode catchment)

collected during dry weather conditions—3 from EPL and 2 from Bode catchments. Plant samples were collected in the immediate vicinity of the sampled rivers. The composite of random plant samples were collected using pre-cleaned scissors and kept in plastic bag. Both plant and water samples were chilled with ice packs during transportation to the UFZ laboratory, and then stored at − 24 °C until analysis.

Chemicals and materials

For sample preparation and analysis, LC–MS grade methanol, formic acid and ammonium formate from Honeywell and LC–MS grade water from Thermo-Fisher were used. For the extraction of plant materials, glass-bottled drinking water (Lauretana, characterized by low contents of minerals) was used. For structural confirmation and quantification, analytical standards at least of 90% purity were obtained from various suppliers (see Additional file 1: Table S2).

Sample extraction and preparation

The collected plant samples were cut into pieces and 0.5 g portion were soaked in 50 mL water in an extraction vessel for 2 h and 30 min. This time period was selected to represent the duration of a typical rain event. The aqueous extract was separated from the solid residue using glass microfiber filters (Whatman GF/A, diameter 47 mm) in vacuum filtration. The filtrates were stored in the freezer for subsequent analysis using LC-HRMS.

Water samples and plant eluates were prepared for direct injection by adding 25 µL of internal standard mix (see Additional file 1: Table S3) containing isotope-labelled compounds (40 ng/L), 25 µL of methanol (LC–MS grade) and 10 µL of ammonium formate buffer (2 M, pH = 3.5) to each 1 mL of sample aliquot. Field, trip and method blanks were treated and analysed exactly in the same way as water samples and plant eluates.

Chemical analysis using LC-HRMS

LC separation was done on a Kinetex C18 EVO column (50 × 2.1 mm, 2.6 µm particle size) using a gradient elution with 0.1% of formic acid (eluent A) and methanol containing 0.1% of formic acid (eluent B) at a flow rate of 300 µL/min. After 1 min of 5% B, the fraction of B was linearly increased to 100% within 12 min and 100% B were kept for 11 min. Subsequently, the column was rinsed for 2 min with a mixture of isopropanol + acetone 50:50/eluent B/eluent A (85%/10%/5%) to remove hydrophobic matrix constituents from the column. Finally, the column was re-equilibrated to initial conditions for 5.7 min. To protect the main column from matrix, a 0.2 µm stainless steel inline filter (Phenomenex) and a Kinetex XB-C18 3 × 5 mm pre-column were used. Aliquots of 100 µL were injected to Thermo Ultimate 3000 LC system (consisting of a ternary pump, auto sampler

and column oven operated at 40 °C) coupled to a quadrupole-orbitrap instrument (Thermo QExactive Plus) using electrospray ionisation (ESI). The spray voltage was 3.8 kV (positive mode), the sheath gas flow rate was 45 a.u., the auxiliary gas flow rate 1 a.u. and the heater temperature 300 °C. Full scan experiments (100–1500 *m/z*) at a nominal resolving power of 140,000 (referenced to *m/z* 200) were conducted in positive ion mode. For structural determination and confirmation, data dependent MS/MS experiments were carried out at nominal resolving power of 35,000. Since many SPMs contain nitrogen functionalities, esters or keto groups ionizing preferably in positive ion mode, we used only positive mode data for the detection and identification of SPMs.

Data handling for qualitative analysis

For data processing, the Thermo raw files acquired were converted to mzML format and centroids with ProteoWizard (version 2.1.0) [25] and imported into MZmine 2.38 [53]. MZmine parameters such as mass detection, chromatogram building smoothing, peak alignment and gap filling were adjusted to get optimal peak detection (for more information see Additional file 2: Table S4) [29, 30, 43]. The transformed peak list was exported as csv file for further processing in MS Excel 2013. To remove noise and background and to reduce false positives, we applied a lower cut-off intensity (10^4) and blank correction. The positive detects were discarded if the peak intensities in the extracted chromatogram were below the threshold intensity (Eq. 1) or if a peak of similar retention time and similar or higher intensities was found in the blank samples. The remaining positive detects were extracted from the peak list and used for further metabolite identification. For performance evaluation of the workflow, 40 isotope-labelled internal standards were spiked to the samples and blanks, which could all be detected by the peak picking procedure in MZmine.

$$I_T = I_{Bav} + 3 \times SD_{Iblank} \quad (1)$$

(I_T threshold intensity, I_{Bav} average intensity of the blanks, SD_{Iblank} standard deviation of intensities of blanks).

Detection and structural elucidation of unknowns

For the identification of unknowns, we engaged a non-target workflow (Additional file 2: Figure S1) consisting of three main steps; first, an empirical approach focused on selecting peaks from vegetation in river waters; therefore, overlapping peaks between plant and river water from adjacent location were extracted from the dataset. In few cases selection of overlapping peaks resulted in inclusion of isobaric compounds rather than identical compounds. If such a peak in water could be identified as an SPM it was accepted in the list despite it was not detected in plant extract. Second, among the overlapping peaks, those with high intensity in plant extracts were subjected to further analysis. By inspecting extracted ion chromatograms (XICs), peaks with broader shape and well unresolved apex were excluded from the candidate list. Then, molecular formulas were evaluated using the Qual Browser of Thermo Xcalibur and searched against freely available compound databases (PubChem, ChemSpider, Phytotoxin (TPPT) and KEGG) for formula query. The number of compounds for a given molecular formula was taken as an indicator for the probability of detection of the compound in river water and for the commercial availability of a reference standard. The isotopic pattern similarity between the computed formula and recorded mass spectra was used for confirmation of the elemental composition. The plausibility of the generated chemical formulas was checked using Seven Golden Rules for heuristic filtering of molecular formulas [31]. Finally, for structural elucidation, the MS/MS spectra of most plausible chemical structure were compared in the spectral libraries mzCloud (https://www.mzcloud.org) and MassBank (https://www.massbank.eu), and supported by high rank structure in in silico fragmentation tools MetFrag (https://msbi.ipb-halle.de/MetFrag/), CSI:Finger ID integrated into SIRIUS 4 [14] and CFM-ID(https://cfmid.wishartlab.com/). For more information on the settings used in in silico fragmenters, see Additional file 2: Tables S5, S6, S7. Peaks without plausible hits from spectral database and in silico fragments were discarded. If commercially available, reference standards were purchased for the most likely structures. MS/MS fragmentation in sample and reference standard with a mass accuracy of 5 ppm and the retention time within a window of 0.1 min were used for structural confirmation. The level of identification for each metabolite structure was reported according to confidence level proposed by [57].

Quantification of the identified PMs

TraceFinder (ThermoFisher Scientific Version 3.2) was used for the quantification of identified SPMs. A series of calibration standards ranging from 1 to 5000 ng/L were prepared. All the calibration standards were treated exactly the same way as river waters and plant extracts. Samples exceeding the highest calibration level were diluted and re-run. The metabolites were quantified using the internal standards with the nearest retention time.

Risk estimates

Since for the plant SPMs detected in this study, no toxicological data are available to conduct risk assessment, tentative risk estimates were based on TTC for non-genotoxic and non-endocrine disrupting compounds of

0.1 µg/L in drinking water. We defined the ratio between measured concentration of the compounds i (ci) and TTC as risk quotient (RQ), and calculated mixture RQs as the sum of individual RQs (Eq. 2) assuming a mixture RQ below one as safe for exposed humans and aquatic organisms.

$$\left(\sum \mathrm{RQ}\right) = \sum \left(c_i / \mathrm{TTC}\right) \quad (2)$$

Results and discussion

Peaks detected in waters and plant extracts

For both water and aqueous plant eluates, the transformation of LC-HRMS output data resulted in a massive dataset (peaks list). After noise, background contaminant and blank correction, 13,000 to 29,000 and 50,000 to 70,000 peaks (defined by *m/z*, retention time and intensity) were considered to be positive detects in river waters and plant extracts, respectively. The positive detects represented organic molecules from all possible sources in the environment—both anthropogenic and natural.

In a first step, we identified peaks common to vegetation and adjacent river water, which ranged from 4900 to 18,500 peaks for the individual pairs (Fig. 2).

For illustration, an aqueous extract of *Galanthus nivalis* and river water from an adjacent location are discussed here. As displayed in Fig. 3, a larger number of common peaks (red spots) were obtained between *Galanthus nivalis* extracts and rain event water samples (Fig. 3—right) than for water samples under dry weather conditions (Fig. 3—left). A similar trend could be observed for all analysed plant-river water pairs. The majority of peaks in plant extracts (green spots) exhibit a higher retention time and thus hydrophobicity than those in water (blue spots).

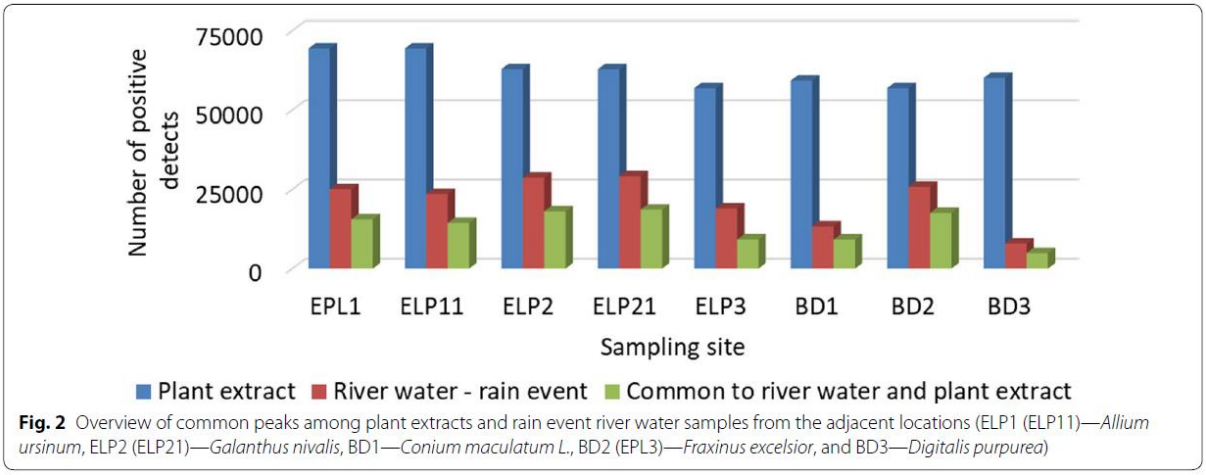

Fig. 2 Overview of common peaks among plant extracts and rain event river water samples from the adjacent locations (ELP1 (ELP11)—*Allium ursinum*, ELP2 (ELP21)—*Galanthus nivalis*, BD1—*Conium maculatum L.*, BD2 (EPL3)—*Fraxinus excelsior*, and BD3—*Digitalis purpurea*)

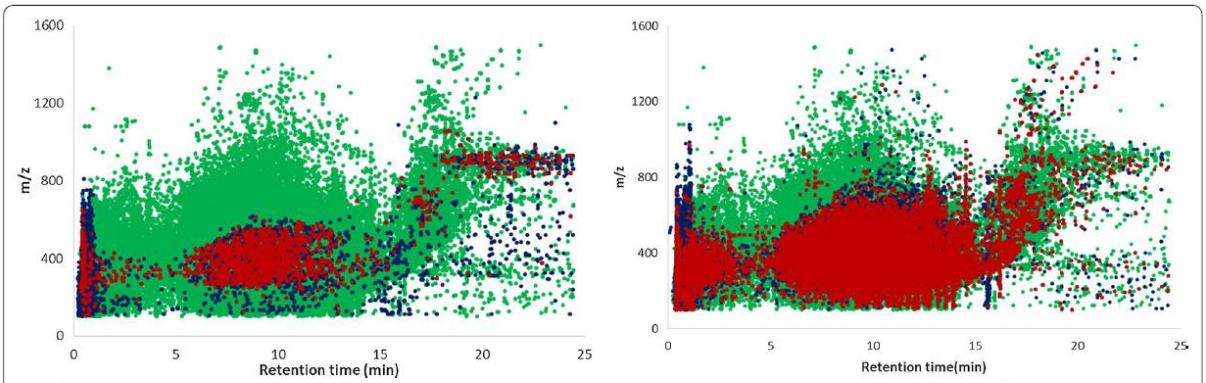

Fig. 3 Overlapping positive detects between *Galanthus nivalis* extract and river water from adjacent location during dry weather condition (left) and rain event (right). (Green spot—positive detects in *Galanthus*, blue spot—positive detects in river water and red spot—positive detects common to both)

The agreement of *m/z* and retention times still allows for different isobaric compounds detected at the same retention time and thus requires further steps to narrow down to common structures.

Peak prioritization and structural identification of metabolites

Prioritization of overlapping peaks

Peaks were prioritized for identification using a stepwise filtering approach demonstrated on the basis of *Galanthus nivalis* and a rain event river water sample from an adjacent location (Fig. 4). After limiting positive detects in both samples to common peaks only (8574), the overlapping peaks were ranked based on intensity in plant extracts and corresponding water samples considering two general assumptions. (1) Peaks with low intensity in plant extracts (selected threshold 10^6) have low probability to enter to river water in a sufficient quantity to be detected. (2) Peaks appearing at higher intensity in river water than in plant extracts are unlikely to originate from the plants. Both criteria were used to exclude peaks of low priority. In our example, this prioritization step reduced the number of peaks to be considered to 1406 which is 8% of the initial peak list (16,594 peaks). Broad peaks with low intensity and not well-defined apex were manually eliminated by inspecting the peak shape. In a next step, the elemental composition of each peak was evaluated based on accurate mass (with an error range given in 5 ppm for exact mass) considering the elements C, H, N, O, P and S—commonly occurring in natural products [9, 54, 64]. Finally, the isotopic fit analysis resulted in 261 (1.5% of initial peaks) tentatively identified candidate peaks.

Identification of unknown SPMs

All 216 peaks selected as candidates were subjected to further identification efforts combining a set of software tools for retrieving possible chemical structure with selection criteria based on database (and software) search and MS/MS fragment consideration as exemplified for two structures below. For a river water sample with high abundance of *Galanthus nivalis* in the catchment, we perceived plausible chemical structure for 54 out of 216 candidate peaks using spectral database search (MassBank and MZcloud) and in silico fragmenters (Metfrag, CSI Finger ID, CFM-ID). By analysing MS/MS fragment, we were able to identify nine of the metabolites (Fig. 4) to confidence level 1—agreement with reference standard based on two orthogonal variables MS2 and retention time [57]. Three more metabolites were also identified to level 1 in the remaining water samples resulting in a total of twelve identified SPMs and other metabolites. The stepwise identification of unknown SPMs will be demonstrated for two examples.

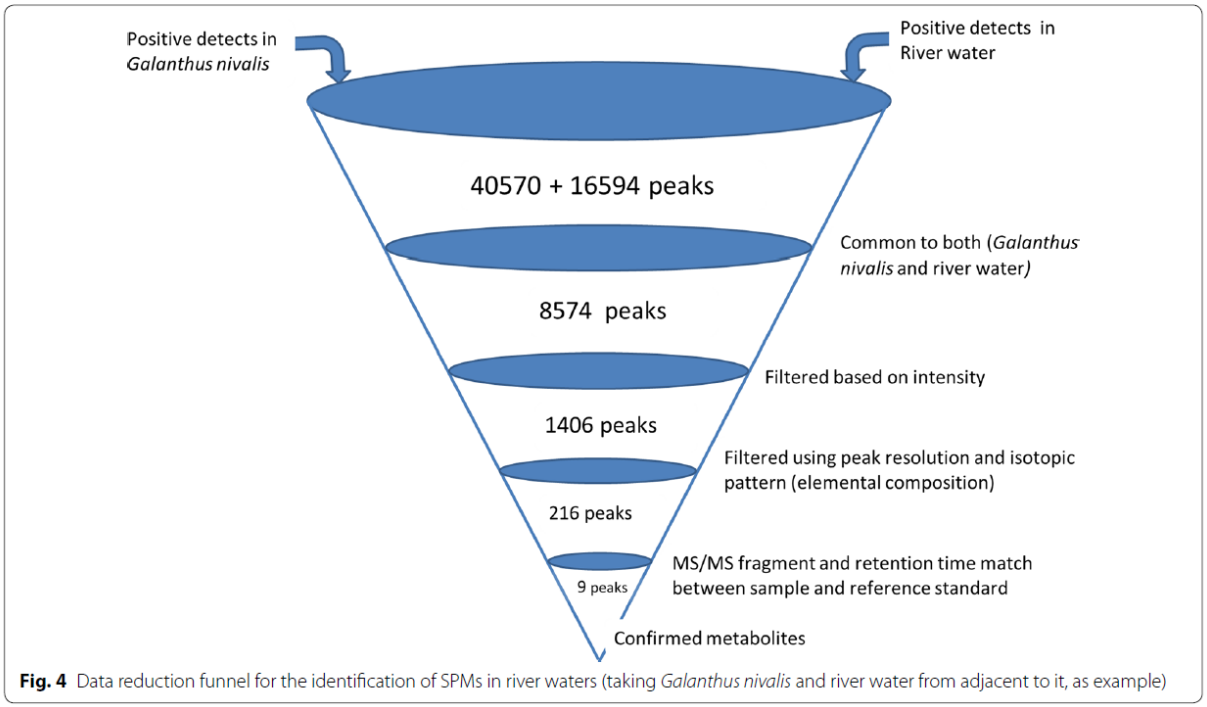

Fig. 4 Data reduction funnel for the identification of SPMs in river waters (taking *Galanthus nivalis* and river water from adjacent to it, as example)

For one of the candidates, the accurate *m/z* of the unknown protonated molecule at a retention time of 0.8 min was determined to be 136.0619 mu. The PubChem search for the elemental composition resulted in five molecular formulas within 5 ppm mass accuracy. The isotopic pattern analysis confirmed the presence of N in the unknown molecule, thus formula not containing N were excluded, which left $C_5H_5N_5$ to be the only potential candidate with 284 registered chemical structures. Furthermore, the data dependent MS/MS fragment ion masses of the unknown molecule were matched with fragmentation pattern of the suggested molecules in the library. Adenine as the compound with the highest spectral match was selected as potential candidate and confirmed with a reference standard based on retention time and MS/MS fragment (see Additional file 2: Figures S2 and S3).

The second accurate mass, chosen for illustration, is 287.0549 mu eluting with a retention time of 10.4 min. Within the set limit, evaluation of the elemental composition using QualBrower of XCalibur resulted in 22 formulas applying a mass error window of 5 ppm. Formulas containing N and S were discarded, since the isotopic pattern analysis of full scan (MS1) spectra did not provide any evidence on the presence of N and S in the candidate molecule. Consequently, the only remaining molecular formula $C_{15}H_{10}O_6$ ($\Delta = -0.085$ ppm) was taken as potential candidate, for which 302 candidate structures were proposed by the database (PubChem). For the determination of the chemical structure, the data dependent MS/MS fragment ion spectrum was submitted to MetFrag, CFM-ID and CSI:finger ID to compare those with in silico predicted spectra for candidate structures retrieved from databases such as PubChem, KNApSAcK, Chemspider and KEGG. Among the structures suggested, the one with highest score and also with highest spectral similarity, namely kaempferol, was selected as plausible candidate structure. This compound could be confirmed in turn with a commercial reference standard based on retention time and MS/MS fragment match (see Additional file 2: Figures S4 and S5). Thus, from the above analysis the suspected unknown molecule was confirmed to be kaempferol.

Following a similar approach, the presence of nicotiflorin, hyperoside, cynaroside (luteolin 7-O-beta-D-glucoside), trifolin (kaempferol-3-O-galactoside), alpinetin, isofraxidin, apiin, guanosine, quercetin and kaempferitrin was confirmed in river waters. The chromatogram and MS/MS spectra for the identified compounds are given in Additional file 2: Figures S6–S25). All the detected metabolites were also obtained in plant extracts, except alpinetin and kaempferitrin, with common peaks detected in water and plant samples but confirmed only in water with isobaric but not identical compounds in the plant extracts. Among the detected plant metabolites, 10 are SPMs, while the nucleic bases adenine and guanosine are components of DNA and RNA and thus not SPMs in a strict sense but subsumed under the same abbreviation. The chemical structures for the identified metabolites are displayed in Fig. 5. See Additional file 2: Table S8 for full information on the identified metabolites in both river water and plant extracts.

Distribution of the identified metabolites in river waters

SPMs of different classes, flavonoids (and their glucosides), coumarins and purine nucleobases were identified and quantified (Fig. 6). In total, the presence of twelve SPMs in river waters from both catchments was confirmed with flavonoids being the predominant class detected. In general, most of the identified metabolites contain one or more phenolic groups representing a class of compounds found most abundantly in vegetation [55]. The identified SPMs have been detected in individual water samples at concentrations up to about 5 µg/L (Fig. 6, and Additional file 2: Table S8). The highest number and concentrations of identified SPMs have been found in two samples (ELP2 and ELP21) from the ELP catchment collected during heavy rain, while in none of the control (dry weather) samples, the identified metabolites were detected (data not shown). This finding supports the hypothesis that rain events drive the leaching of SPMs to surface water.

Most SPMs were detected in water samples from both catchments, with the exception of alpinetin, hyperoside, kaempferitrin and quercetin which were detected in the ELP catchment only. Among the detected SPMs, adenine and isofraxidin were obtained at high frequency in both water samples and plant extracts. This has been followed by cynaroside in water samples and trifolin in plant extracts (Table 1 and Additional file 2: Figure S26). In river waters, SPMs were detected in an overall concentration range of 0.02 to 5.1 µg/L (Fig. 6, Table 1).

The purine bases adenine and guanosine were detected at concentration range of 0.4–4.0 µg/L and 35–189.5 µg/g in water samples and plant extracts, respectively (Table 1). Adenine is an aromatic base found in both DNA and RNA of living organisms. The compounds were previously isolated from a variety of plants (e.g., maize, tea and coffee plants) [4, 59]. Guanosine was reported to have neurotrophic and neuroprotective effects, evidenced from rodent and cell models study in vivo at 7.5 mg/kg [10, 37, 52].

Flavonoids, a class of natural compounds widely distributed in plants, including kaempferol and quercetin were detected in several water samples and plant extracts from ELP and one from Bode catchment. Quercetin was

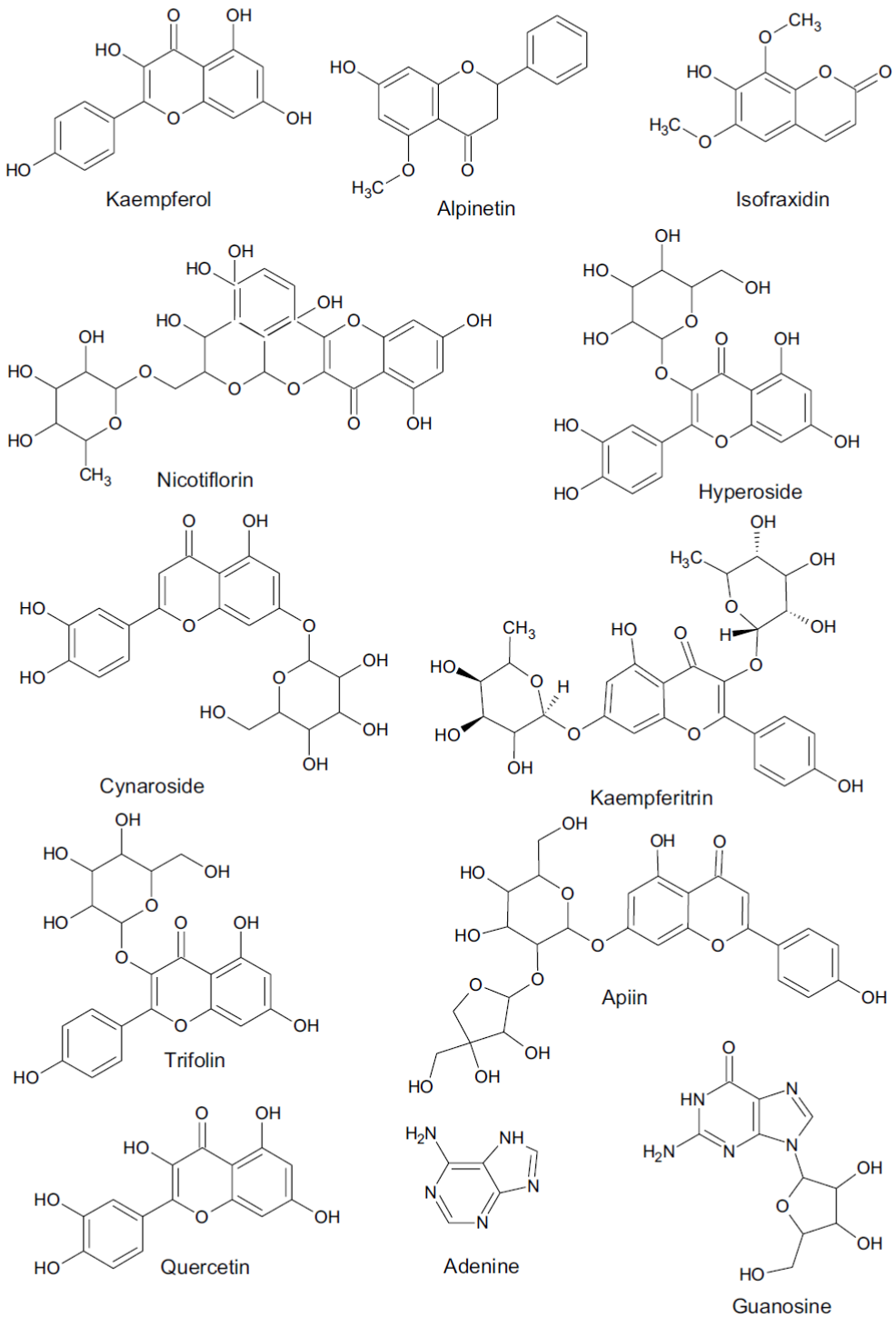

Fig. 5 The chemical structure of identified SPMs in river waters

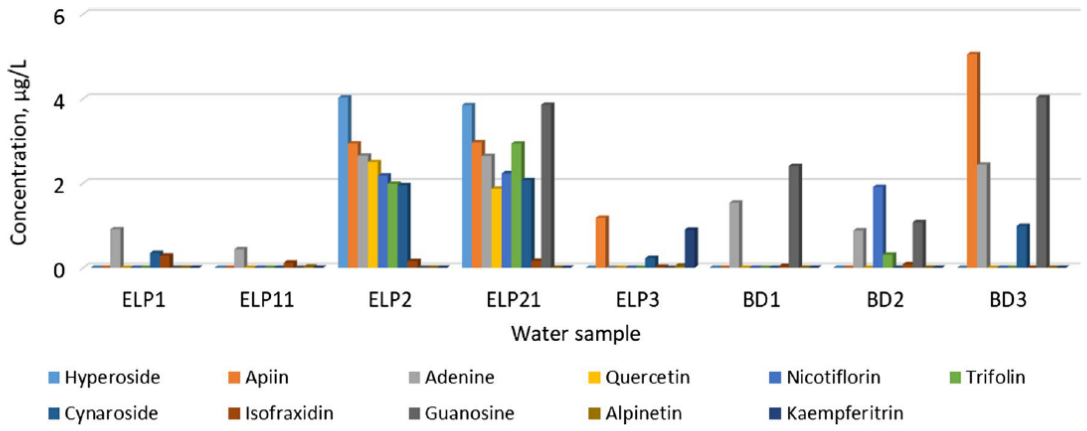

Fig. 6 Distribution of detected metabolites in river waters

Table 1 Concentration of the detected metabolites in both river waters and plant extracts

Metabolites	Formula	CAS no	Precursor ion (*m/z*)	Retention time (min)	MDL (µg/L)	River water		Plant extracts	
						Detection frequency	Concentration range (min–max, µg/L)	Detection frequency	Aqueous extractable concentration range (min–max, µg/g)
Adenine	$C_5H_5N_5$	73-24-5	136.0619	0.8	0.2	7	0.4–2.6	5	35.0–59.3
Alpinetin	$C_{16}H_{14}O_4$	36052-37-6	271.0962	10.3	0.004	2	0.023–0.050	0	ND
Apiin	$C_{26}H_{28}O_{14}$	26544-34-3	565.1547	9.1	0.5	4	1.2–5.1	1	21.7
Cynaroside	$C_{21}H_{20}O_{11}$	5373-11-5	449.1073	8.6	0.050	5	0.2–2.1	3	11.1–50.6
Guanosine	$C_{10}H_{13}N_5O_5$	118-00-3	284.0984	1.0	0.2	4	1.1–4.0	5	42.8–189.5
Hyperoside	$C_{21}H_{20}O_{12}$	482-36-0	465.1017	8.6	0.3	2	3.8–4.0	2	18.9–22.6
Isofraxidin	$C_{11}H_{10}O_5$	486-21-5	223.0599	7.4	0.014	7	0.020–0.300	5	0.01–16.8
Kaempferitrin	$C_{27}H_{30}O_{14}$	482-38-2	579.171	9.3	0.050	1	0.9	0	ND
Kaempferol	$C_{15}H_{10}O_6$	520-18-3	287.0548	10.6	–	3	NQ	3	NQ
Nicotiflorin	$C_{27}H_{30}O_{15}$	17650-84-9	595.165	9.2	0.2	3	1.9–2.2	1	88.0
Quercetin	$C_{15}H_{10}O_7$	117-39-5	303.0496	8.6	0.6	2	1.9–2.5	2	54.6–74.7
Trifolin	$C_{21}H_{20}O_{11}$	23627-87-4	449.1073	9.1	0.2	3	0.3–2.9	4	25.0–36.0

NQ not quantified, *ND* not detected, *MDL* method detection limit

obtained at an average concentration of 2 µg/L. Besides their potential positive effects such as antiproliferative, chemopreventive, and anti-inflammatory activities [35], kaempferol and quercetin inhibit the acetylcholinesterase (AChE) activity in vitro at IC_{50} of approximately 32 and 4.7 mg/L, respectively [44, 48, 51, 65]. In vivo study, quercetin demonstrated toxic and carcinogenic effects in the kidney of male rats at doses above 40 mg/kg [13, 15].

The flavanone alpinetin and the glycosyloxyflavone kaempferitrin (a 3,7-dirhamnoside of kaempferol) were obtained in river waters from ELP, but not in the investigated plant extracts (despite overlapping peaks by isobaric compounds). However, the metabolites were previously reported from a variety of other plants in the environment—alpinetin from genus *Alpinia* (flowering plants) and kaempferitrin from *Lathyrus* (a genus in the legume family Fabaceae) [2, 12, 27, 38, 63]. In the present study, no evidence was obtained for the presence of such plants along the investigated rivers. The measured concentration of kaempferitrin was 0.9 µg/L, while alpinetin was present in concentrations of 23 and 50 ng/L. Besides its antibacterial and anti-inflammatory activities, alpinetin exhibited vasorelaxant effects on rat at a mean concentration (IC_{50}) of about 7.4 mg/L in in vitro study [63]. It also showed potential effects in downregulating the immune system in mice [17]. A study by Zhang

et al. showed that kaempferitrin competitively inhibited human liver microsomal Cytochrome P450 1A2 activity at an IC_{50} of 11 mg/L in vitro [66].

The glycosyloxyflavone apiin was measured at a high concentration (5 µg/L) in a water sample from the Bode catchment but was also obtained in two water samples from ELP at an average concentration of 2.9 µg/L. Another flavonoid glucoside, namely nicotiflorin (kaempferol 3-O-rutinoside) was obtained in rivers from both catchments—two from ELP and one from Bode catchment—at an average concentration of 2 µg/L. However, both metabolites were detected only in one plant extract each—apiin in *Digitalis purpurea* and nicotiflorin in *Fraxinus excelsior* from Bode catchment, though, *Fraxinus excelsior* is a characteristic plant in the ELP floodplain forest, too. The detection of apiin in ELP water samples indicates leaching also from other frequently occurring plant species (not considered in this work) including Apiaceae [2] and stinging nettle (*Urtica dioica*) [50]. In vitro, apiin displayed anti-inflammatory activity at IC_{50} of 49 mg/L [40]. Nicotiflorin has many interesting pharmacological activities, such as decreasing arterial blood pressure and heart rate and hepatoprotective effects in mice in vivo [21]. It was found to protect against memory dysfunction and oxidative stress in multi-infarct dementia model rats at 30 mg/kg in vivo [21, 26].

In only two water samples from ELP, an average concentration of 3.9 µg/L was registered for hyperoside (a quercetin-3-O-D-galactoside). It was also detected in substantial concentrations in plant extracts (*Fraxinus excelsior* and *Galanthus*) from close vicinity, from which it could be emitted (Additional file 2: Table S8). It may have potential as a therapeutic agent for the treatment of liver fibrosis [61]. It improves cardiac function and prevents the development of cardiac hypertrophy via AKT signalling at concentration of about 4.6 mg/L in vitro [62]. Hyperoside, at concentrations 10 mg/kg in vivo, was found to present a depressor effect on the central nervous system as well as an antidepressant-like effect in rodents which is, at least in part, mediated by the dopaminergic system [19]. The water-extractable hypersoside from *Hypericum* species demonstrated an acetylcholinesterase inhibition effect at IC_{50} of 66 mg/L [23].

Cynaroside and trifolin glycosyloxyflavones in water samples occurred at concentrations ranging from 0.2 to 2.1 and 0.3–2.9 µg/L, respectively (Table 1 and Fig. 6). The former was identified in five water samples—four from ELP and one from the Bode catchment, while the later was in three samples—two from Leipzig and one from Bode catchment. Both metabolites were also detected in plant extracts from both catchments. Cynaroside shown to cause a prominent anti-oxidant effect, inhibiting lipid and protein oxidation. In vitro, it also displayed inhibitory effects on human liver cytochrome P450 (CYP) isoforms with an IC_{50} value of 7 mg/L [60]. Trifolin (kaempferol-3-O-galactoside), which is a galactose-conjugated flavonol, exhibits antifungal and anticancer effects at IC_{50} value of about 50 mg/L in vitro [39].

The coumarin, isofraxidin was obtained at an average concentration of 0.03 µg/L in two water samples from each location. In the rest of the water samples, except one from Bode catchment, it was found at an average concentration of 0.2 µg/L. The SPM was quantified in all the plant extracts—the highest being in *Fraxinus excelsior*, a characteristic tree along the rivers in both catchments. Apart from its numerous pharmacological activity such as antioxidant and anti-inflammatory, isofraxidin inhibited human liver cytochrome P450 (CYP) isoforms in vitro with an IC_{50} of about 3 mg/L [58].

Toxic risk estimation

The SPMs have been detected in water samples not as individual compounds but in mixtures of at least three SPMs co-occurring at all sites, while at two samples, even nine metabolites were detected (Fig. 7a). Thus, a preliminary mixture RQ based on a TTC of 0.1 µg/L exceeded 5

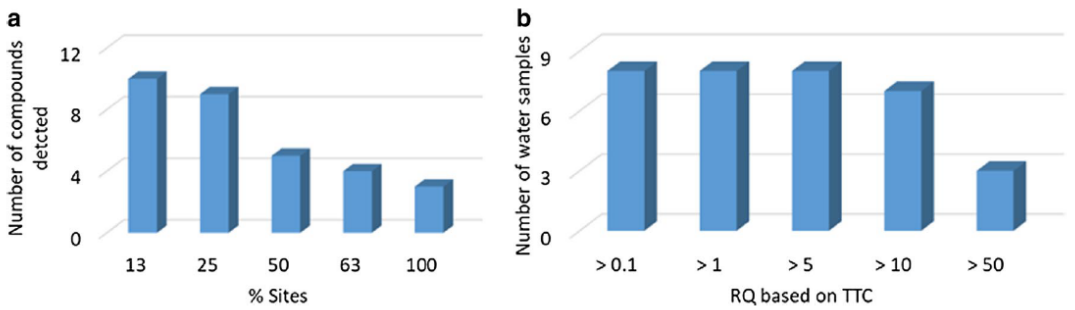

Fig. 7 **a** Co-occurrence of detected metabolites in between sites and **b** the number of samples exceeding mixture risk quotient (RQ) levels of metabolites

(and thus also 1 at all the sites), while at 7 sites, a value of 10 and at 3 sites even a value of 50 was exceeded (Fig. 7b). Individual concentrations of the detected SPMs, except isofraxidin (in three water samples) and alpinetin, were also above the TTC. Thus, toxic risks by individual SPMs and mixtures thereof and a contribution to overall toxicity of surface waters cannot be excluded and demand for additional efforts in hazard characterization.

Conclusion

In this study, for the first time a novel approach has been applied to associate unknown peaks of high intensity in LC-HRMS NTS to SPMs from surrounding vegetation by focusing on peaks overlapping between river water and aqueous plant extracts. A high number of peaks has been found in this overlap suggesting a significant impact of vegetation on chemical mixtures detectable in surface waters. In total, 12 SPMs and other metabolites could be identified including flavonoids, flavonoid glucoside, coumarins and purine bases with flavonoids as the predominant compounds. SPMs are produced by many plants and in surface water their individual concentration may reach up to 5 µg/L exceeding the TTC level (0.1 µg/L) for non-genotoxic and non-endocrine disrupting chemicals in drinking water. Although this finding does not necessarily indicate toxic risk to aquatic organisms it may illustrate the relatively high concentrations at which a contribution to mixture toxicity cannot be excluded. There might be possible contribution of these compounds to the effects sometimes detected with the effect-based monitoring tools even in natural and apparently pristine areas. Thus, this should be considered to explain discrepancies between expected effects by anthropogenic chemicals found in a water sample and detections with effect-based methods. Impacts of SPMs on quality of drinking water abstracted from natural water resources cannot be excluded. However, due to the lack of aquatic toxicity data for SPMs and extremely scarce exposure data, no reliable risk assessment and prioritization of SPMs for monitoring and assessment can be performed. Thus, SPMs should be included increasingly into chemical monitoring of surface waters to collect exposure data on a larger scale complemented with toxicity testing of compounds occurring frequently or in high concentrations. Substantial toxicity of individual compounds to mammals as reported above may also trigger hazard assessment of SPMs found in surface waters. The present study clearly indicates that identified compounds represent only the tip of the iceberg of possibly toxic SPMs in water resources. Thus, NTS-based approaches should be increasingly applied to understand complex mixtures of synthetic contaminants and SPMs.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12302-020-00415-5.

Additional file 1: Table S1: Information on sampling site for river water and plant species. **Table S2.** Analytical standards used. **Table S3.** Internal standards used for the chemical analysis (ESIpos).

Additional file 2: Table S4. Setting for MZmine data processing. **Table S5.** Setting used in CSI:Finger ID for in silico fragment pattern prediction. **Table S6.** Setting used in MetFrag for in silico fragment pattern prediction. **Table S7.** Setting used in CFM ID for in silico fragment pattern prediction. **Table S8.** Concentration of detected metabolites in both water samples and aqueous plant elutriates. **Figure S1.** Work flow for the non-target detection of SPMs in river waters. **Figure S2.** Extracted ion chromatograms of adenine in reference standard, water sample and plant (*Galanthus nivalis*) elutriates. NL: signal intensity at 100%. **Figure S3.** MS/MS spectra (HCD fragmentation at 55 a.u.) of adenine in a reference standard, water sample and plant (*Galanthus nivalis*) elutriates. **Figure S4.** Extracted ion chromatograms of kaempferol in reference standard, water sample and plant (*Galanthus nivalis*) elutriates. (NL: signal intensity at 100%). **Figure S5.** MS/MS spectra (HCD fragmentation at 55 a.u.) of kaempferol in a reference standard, water sample and plant (*Galanthus nivalis*) elutriates. **Figure S6.** Extracted ion chromatograms of apiin in reference standard, water sample and plant (*Digitalis purpurea*) elutriates. NL: signal intensity at 100%. **Figure S7.** MS/MS spectra (HCD fragmentation at 45 a.u.) of apiin in a reference standard, water sample and plant (*Digitalis purpurea*) elutriates. **Figure S8.** Extracted ion chromatograms of hyperoside in reference standard, water sample and plant (*Galanthus nivalis*) elutriates. NL: signal intensity at 100%. **Figure S9.** MS/MS spectra (HCD fragmentation at 55 a.u.) of hyperoside in a reference standard, water sample and plant (*Galanthus nivalis*) elutriates. **Figure S10.** Extracted ion chromatograms of nicotiflorin in reference standard, water sample and plant (*Fraxinus excelsior*) elutriates. NL: signal intensity at 100%. **Figure S11.** MS/MS spectra (HCD fragmentation at 45 a.u.) of nicotiflorin in a reference standard, water sample and plant (*Fraxinus excelsior*) elutriates. **Figure S12.** Extracted ion chromatograms of cynaroside in reference standard, water sample and plant (*Galanthus nivalis*) elutriates. NL: signal intensity at 100%. **Figure S13.** MS/MS spectra (HCD fragmentation at 45 a.u.) of cynaroside in a reference standard, water sample and plant (*Galanthus nivalis*) elutriates. **Figure S14.** Extracted ion chromatograms of isofraxidin in reference standard, water sample and plant (*Fraxinus excelsior*) elutriates. NL: signal intensity at 100%. **Figure S15.** MS/MS spectra (HCD fragmentation at 55 a.u.) of isofraxidin in a reference standard, water sample and plant (*Fraxinus excelsior*) elutriates. **Figure S16.** Extracted ion chromatograms of kaempferitrin in reference standard and water sample. NL: signal intensity at 100%. **Figure S17.** MS/MS spectra (HCD fragmentation at 45 a.u.) of kaempferitrin in a reference standard and water sample. **Figure S18.** Extracted ion chromatograms of alpinetin in reference standard and water sample. NL: signal intensity at 100%. **Figure S19.** MS/MS spectra (HCD fragmentation at 45 a.u.) of alpinetin in a reference standard and water sample. **Figure S20.** Extracted ion chromatograms of quercetin in reference standard, water sample and plant (*Fraxinus excelsior*) elutriates. NL: signal intensity at 100%. **Figure S21.** MS/MS spectra (HCD fragmentation at 55 a.u.) of quercetin in a reference standard, water sample and plant (*Fraxinus excelsior*) elutriates. **Figure S22.** Extracted ion chromatograms of guanosine in reference standard, water sample and plant (Digitalis purpurea) elutriates. NL: signal intensity at 100%. **Figure S23.** MS/MS spectra (HCD fragmentation at 45 a.u.) of guanosine in a reference standard, water sample and plant (Digitalis purpurea) elutriates. **Figure S24.** Extracted ion chromatograms of trifolin in reference standard, water sample and plant (*Galanthus nivalis*) elutriates. NL: signal intensity at 100%. **Figure S25.** MS/MS spectra (HCD fragmentation at 45 a.u.) of trifolin in a reference standard, water sample and plant (*Galanthus nivalis*) elutriates. **Figure S26.** Distribution of detected metabolites in the aqueous plant elutriates.

Abbreviations

LCHRMS: Liquid chromatography coupled to high resolution mass spectrometry; SPMs: Secondary plant metabolites; ND: Not detected; NQ: Not quantified;

SI: Supplementary information; NTS: Non target screening; ELP: Elster, Luppe and Pleiße catchment; BD: Bode catchment; TTC: Threshold for toxicological concern; RQ: Risk quotient; MDLs: Method detection limit.

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Author's contributions
MYN: Conceptualization, investigation, experimental analysis, data evaluation and visualization, writing (original draft) and editing. MK: Conceptualization, investigation, writing—review and editing; WB: Conceptualization, supervision, writing—review and editing. All authors read and approved the final manuscript.

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Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Supplementary Information for

Non-target screening for detecting the occurrence of plant metabolites in river waters

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Table S1: Information on sampling site for river water and plant species

ID	River	Catchment	Sampling date	Coordinate	Target plant
BD1	Barrenbach	Bode	02.07.2019	51°51'51.7"N; 10°49'55.8"E	*Conium maculatum L*
BD2	Getel	Bode	02.07.2019	51°45'49.9"N; 11°19'50.9"E	*Fraxinus excelsior*
BD3	Drangetalwasser	Bode	02.07.2019	51°47'03.8"N; 10°44'16.4"E	*Digitalis purpurea*
ELP1	Tributary to Elsterflutbett	ELP	18.03.2019	51°18'28.1"N; 12°21'05.6"E	*Fraxinus excelsior, Allium ursanium, Galanthus nivalis*
ELP11	Tributary to Elsterflutbett	ELP	15.04.2019	51°18'28.1"N; 12°21'05.6"E	*Fraxinus excelsior, Allium ursanium, Galanthus nivalis*
ELP2	Paußnitz	ELP	19.06.2019	51°18'34.8"N ; 12°20'51.9"E	*Fraxinus excelsior, Allium ursanium, Galanthus nivalis*
ELP21	Paußnitz	ELP	19.06.2019	51°18'56.0"N; 12°21'21.9"E	*Fraxinus excelsior, Allium ursanium, Galanthus nivalis*
ELP3	Tributary to Elsterflutbett	ELP	19.06.2019	51°18'54.6"N; 12°21'14.5"E	*Fraxinus excelsior, Allium ursanium, Galanthus nivalis*

Table S2: Analytical standards used

SN	Metabolites	Formula	CAS No	Supplier	Purity (%)
1	Adenine	$C_5H_5N_5$	73-24-5	Sigma-Aldrich	≥99
2	Alpinetin	$C_{16}H_{14}O_4$	36052-37-6	Phytolab	≥95
3	Apiin	$C_{26}H_{28}O_{14}$	26544-34-3	Phytolab	≥95
4	Cynaroside	$C_{21}H_{20}O_{11}$	5373-11-5	Geyer/J&K	98
5	Guanosine	$C_{10}H_{13}N_5O_5$	118-00-3	Sigma-Aldrich	≥98
6	Hyperoside	$C_{21}H_{20}O_{12}$	482-36-0	Roth	≥95
7	Isofraxidin	$C_{11}H_{10}O_5$	486-21-5	Sigma-Aldrich	≥98
8	Kaempferitrin	$C_{27}H_{30}O_{14}$	482-38-2	Phytolab	≥95
9	Kaempferol	$C_{15}H_{10}O_6$	520-18-3	ABCR	98
10	Nicotiflorin	$C_{27}H_{30}O_{15}$	17650-84-9	Sigma-Aldrich	≥98
11	Quercetin	$C_{15}H_{10}O_7$	117-39-5	Roth	≥98
12	Trifolin	$C_{21}H_{20}O_{11}$	23627-87-4	Sigma	≥90

Table S3: Internal standards used for the chemical analysis (ESIpos)

ID	Compound name	Monoisotopic mass	Used ions (ESI+)		
			M+	M+H+	M+NH4+
IS03	IS03_Mono-isobutylphthalate-D4	226.1143		227.1216	
IS04	IS04_Creatinine-D3	116.0777		117.085	
IS05	IS05_Diazinon-D10	314.1638		315.1711	
IS06	IS06_Benzophenone-3-D5	233.11		234.1173	
IS07	IS07_p-Toluene-sulfonamide-D4	175.0605		176.0678	193.0933
IS10	IS10_1-Naphthol-D7	151.1015			
IS13	IS13_Cotinine-D3	179.1138		180.1211	
IS16	IS16_Bisphenol A D16	244.2155			
IS17	IS17_Diglyme-D6	140.132		141.1392	
IS18	IS18_4-Nitrophenol-D4	143.0521			
IS19	IS19_Chlormequat-D9	131.1296	131.13		
IS22	IS22_Carbamazepine-D10	246.1577		247.165	
IS23	IS23_Triclosan-D3	290.97			
IS24	IS24_Atrazine-13C3	218.1038		219.1111	
IS25	IS25_Estradiol-D3	275.1965			
IS27	IS27_4-Nonylphenol-D4	224.2078			
IS28	IS28_Benzotriazole-D4	123.0735		124.0807	
IS29	IS29_Carbendazim-D4	195.0946		196.1019	
IS30	IS30_Tri-n-butylphosphate-D27	293.3342		294.3414	
IS31	IS31_DEET-D7	198.175		199.1822	
IS37	IS37_Metolachlor-D6	289.1716		290.1788	
IS38	IS38_Isoproturon-D3	209.1607		210.168	
IS39	IS39_Mecoprop-D3	217.0585			
IS40	IS40_Diclofenac-D4	299.0418		300.0491	
IS41	IS41_Caffeine-D3	197.0992		198.1065	
IS42	IS42_Clarithromycin-D3	750.4957		751.503	
IS43	IS43_Desisopropylatrazine-D5	178.0782		179.0855	
IS44	IS44_Decyltrimethylammonium-D30	230.4256	230.43		
IS46	IS46_Laurylsulfate-D25	291.3121			
IS47	IS47_Atenolol-D7	273.207		274.2143	
IS48	IS48_Progesterone-D9	323.2811		324.2883	
IS49	IS49_Verapamil-D6	460.3208		461.3281	
IS50	IS50_Bezafibrate-D4	365.1332		366.1405	
IS51	IS51_Sulfamethoxazole-D4	257.0772		258.0845	
IS54	IS54_Acesulfame-D4	167.019			
IS55	IS55_Tebuconazole-D9	316.2016		317.2089	
IS56	IS56_Hydrochlorothiazide-13C6	302.9846			
IS57	IS57_Imidacloprid-D4	259.0774		260.0847	
IS62	IS62_Bentazone-D6	246.09452			
IS63	IS63_Cyclamate-D11	190.13066			

Table S1: Setting for MZmine data processing.

	Parameters	**Mass detection**	**Chromatogram building**	**Chromatogram deconvolution**	**Join aligner**	**Gap filling**	**Target annotation (identification)**
Peak detection	Mass detector	Centroid					
	Noise level	5.00E+3					
	MS level	1.0					
	Group intensity threshold		1.00E+4				
	Min height intensity		5.00E+03				
	m/z tolerance		0.001				
	Algorithm			Local minimum search			
	Chromatographic threshold (%)			60.0			
	Search minimum in retention time range			0.1			
	Minimum relative height (%)			30			
	Minimum absolute height			5.0E+4			
	Min ratio of peak top/edge			2.3			
	Peak duration range (min)			0.1-0.5			
Peak alignment and identification	m/z tolerance				0.001	0.001	0.001
	Weight for m/z tolerance				70		
	Retention time tolerance (absolute, min)				0.3	0.15	0.5
	Weight for RT				30		
	Intensity tolerance (%)					30.00	
	RT range						
	Adducts						M+H+, M+Na+, M+NH4+,

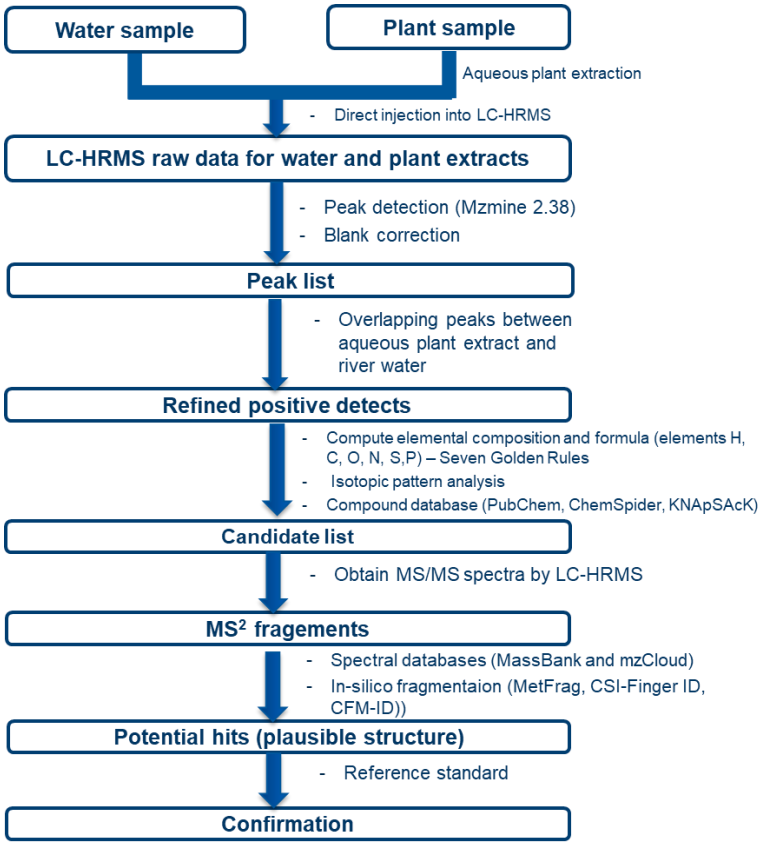

Figure S1: Work flow for the non-target detection of SPMs in river waters.

Table S2: Setting used in CSI:Finger ID for in-silico fragment pattern prediction.

	Parameters	**Inputs**
Input data	Precursor mass	m/z of parent ion
	Possible ionization	$[M]^+$, $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$
	MS2 Spectrum	MS/MS Fragment ion mass extracted from Xcalibur (m/z and intensity
Elements allowed in Molecular formula	Elements	C,H,O,N,S,P
	Mass tolerance	5 ppm
CSIFingerID-Structure Elucidation	Search in (database)	PubChem, KEGG, Natural products, KNApSAcK
	Molecular formula	Computed formula for the precursor mass

Table S3: Setting used in MetFrag for in-silico fragment pattern prediction

	Parameters	**Inputs**
Database setting	Parent ion (m/z)	Accurate mass (mu) of the unknown
	Database	PubChem / KEGG
	Adducts	$[M]^+$, $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$
	Mass tolerance	5 ppm
	Formula	Molecular formula computed for the parent mass
Candidate filter and Score setting	Candidate filter	Element inclusion - C,H,N,O,P,S Filter Type - Optional
	MetFrag Scoring Terms	MetFrag score
		Spectral Similarity (MoNA) score
		Exact Spectral Similarity (MoNA) score
Fragmentation setting and processing	Relative mass deviation to match generated against MS/MS peaks	MZppm = 5 MZabs = 0.001
	Tree depth	2 (default value)
	Mode (Adduct)	$[M]^+$, $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$
	MS/MS Peak list	LC-HRMS recorded MS/MS fragment data

Table S4: Setting used in CFM ID for in-silico fragment pattern prediction.

	Parameters	**Inputs**
Candidate	Spectra type	ESI
	Ion Mode	Positive
	Adduct Type	$[M]^+$, $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$
	Parent ion mass	Precursor mass (m/z)
	Mass tolerance	5ppm
	Databases	MassBank, KEGG, DrugBank
Spectral	Input spectra text	MS/MS fragment data extracted from Xcalibur
Scoring	Default	DotProduct + Metadata

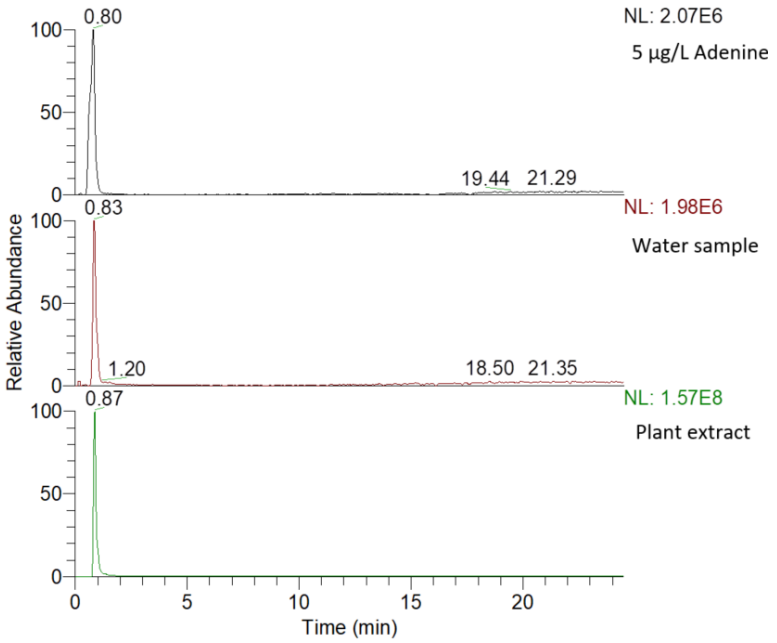

Figure S2: Extracted ion chromatograms of adenine in reference standard, water sample and plant (*Galanthus nivalis*) elutriates. NL: signal intensity at 100%.

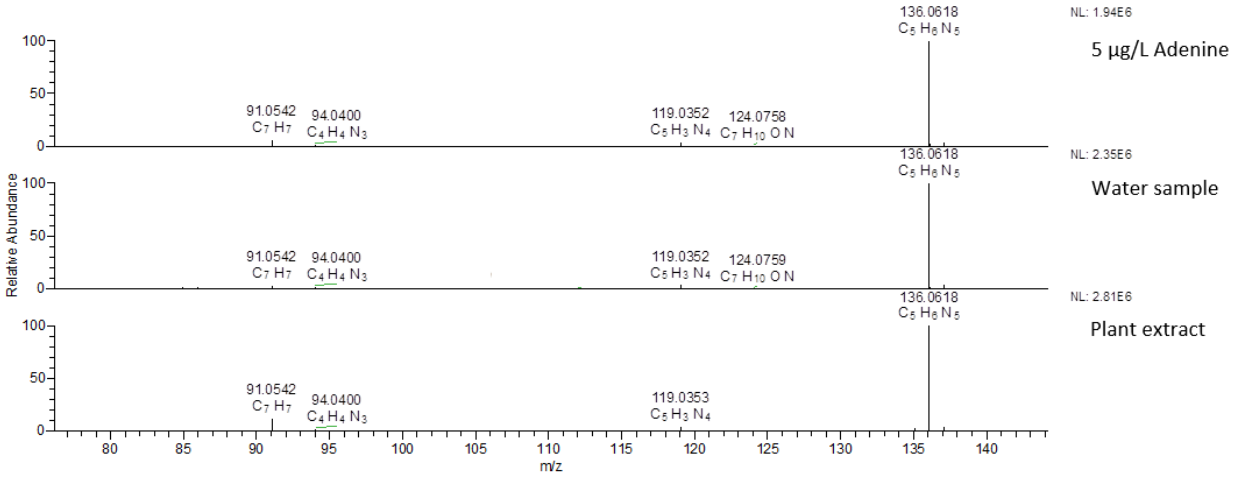

Figure S3: MS/MS spectra (HCD fragmentation at 55 a.u.) of adenine in a reference standard, water sample and plant (*Galanthus nivalis*) elutriates.

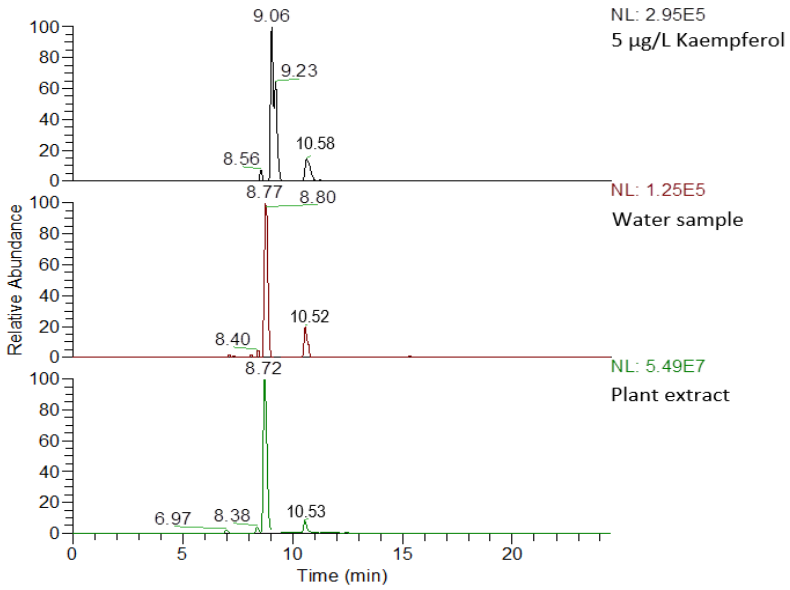

Figure S4: Extracted ion chromatograms of kaempferol (RT = 10.58 min) in reference standard, water sample and plant (*Galanthus nivalis*) elutriates. (NL: signal intensity at 100%).

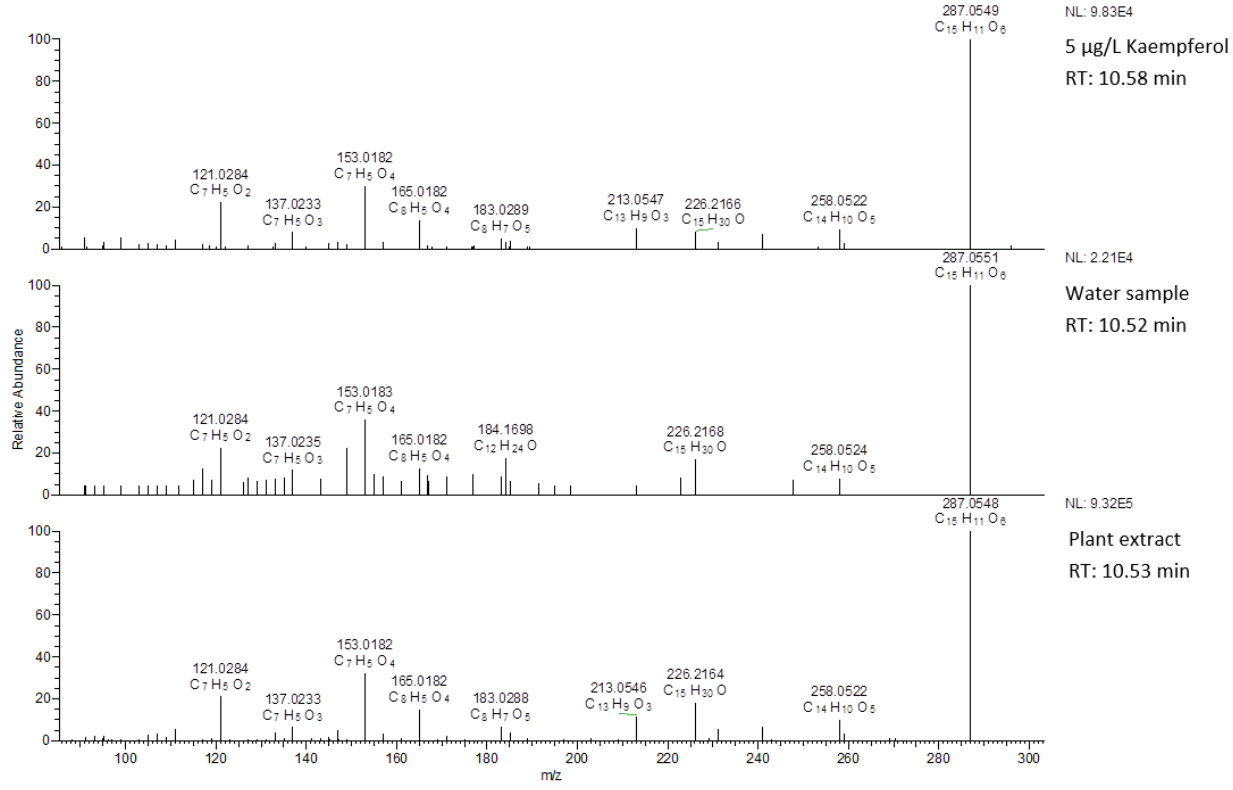

Figure S5: MS/MS spectra (HCD fragmentation at 55 a.u.) of kaempferol in a reference standard, water sample and plant (*Galanthus nivalis*) elutriates.

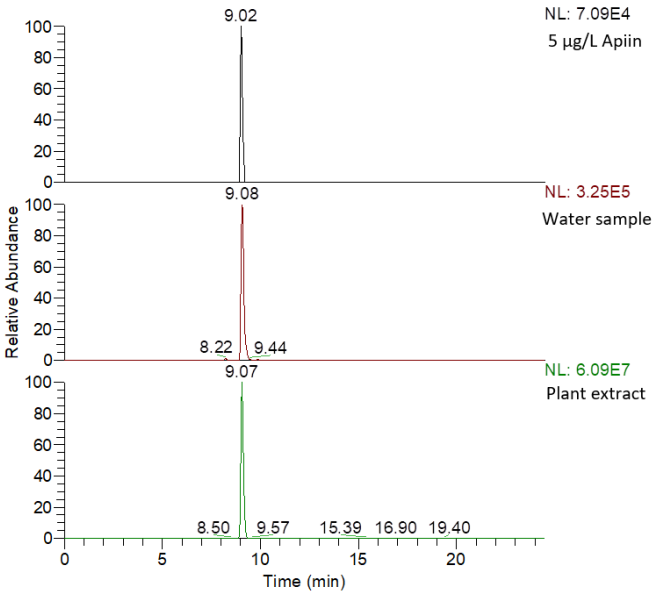

Figure S6: Extracted ion chromatograms of apiin in reference standard, water sample and plant (*Digitalis purpurea*) elutriates. NL: signal intensity at 100%.

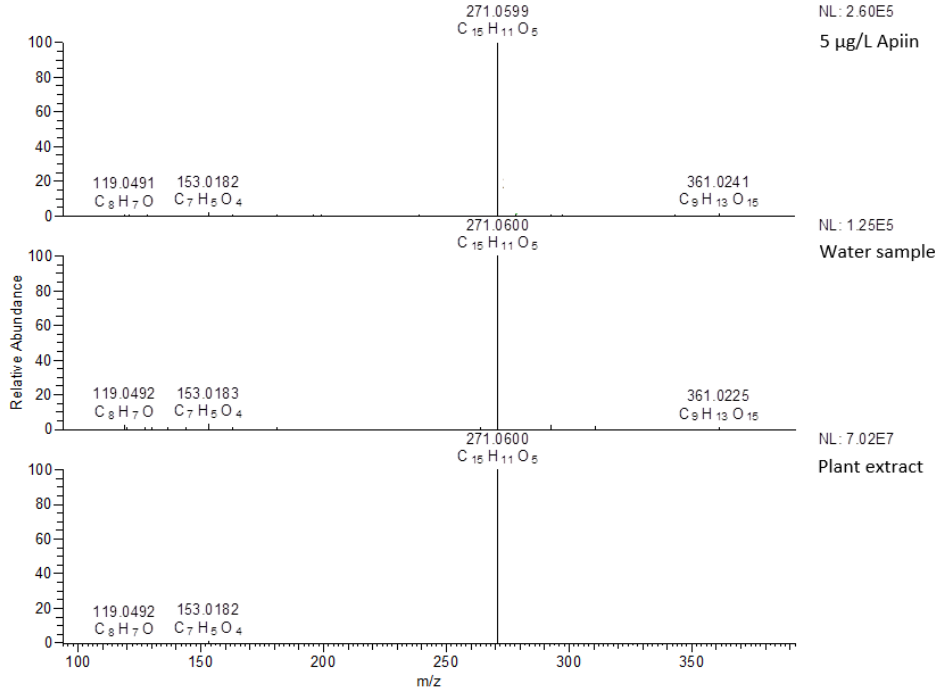

Figure S7: MS/MS spectra (HCD fragmentation at 45 a.u.) of apiin in a reference standard, water sample and plant (*Digitalis purpurea*) elutriates.

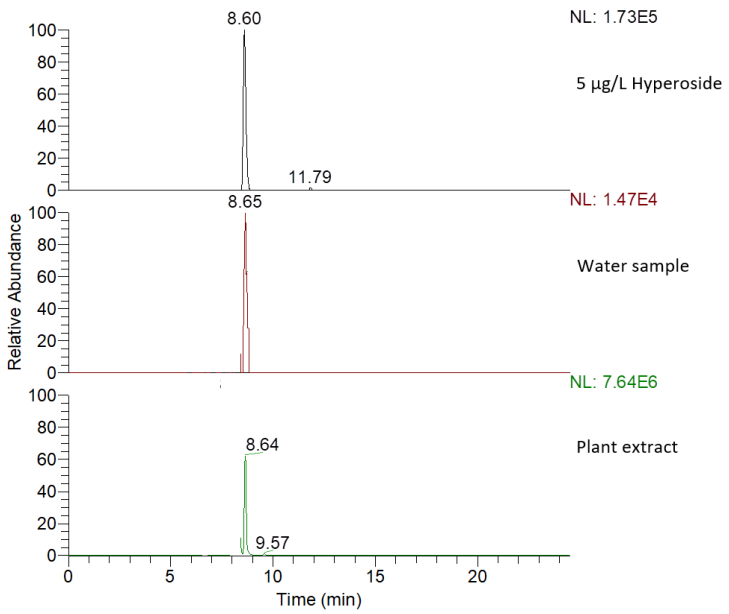

Figure S8: Extracted ion chromatograms of hyperoside in reference standard, water sample and plant (*Galanthus nivalis*) elutriates. NL: signal intensity at 100%.

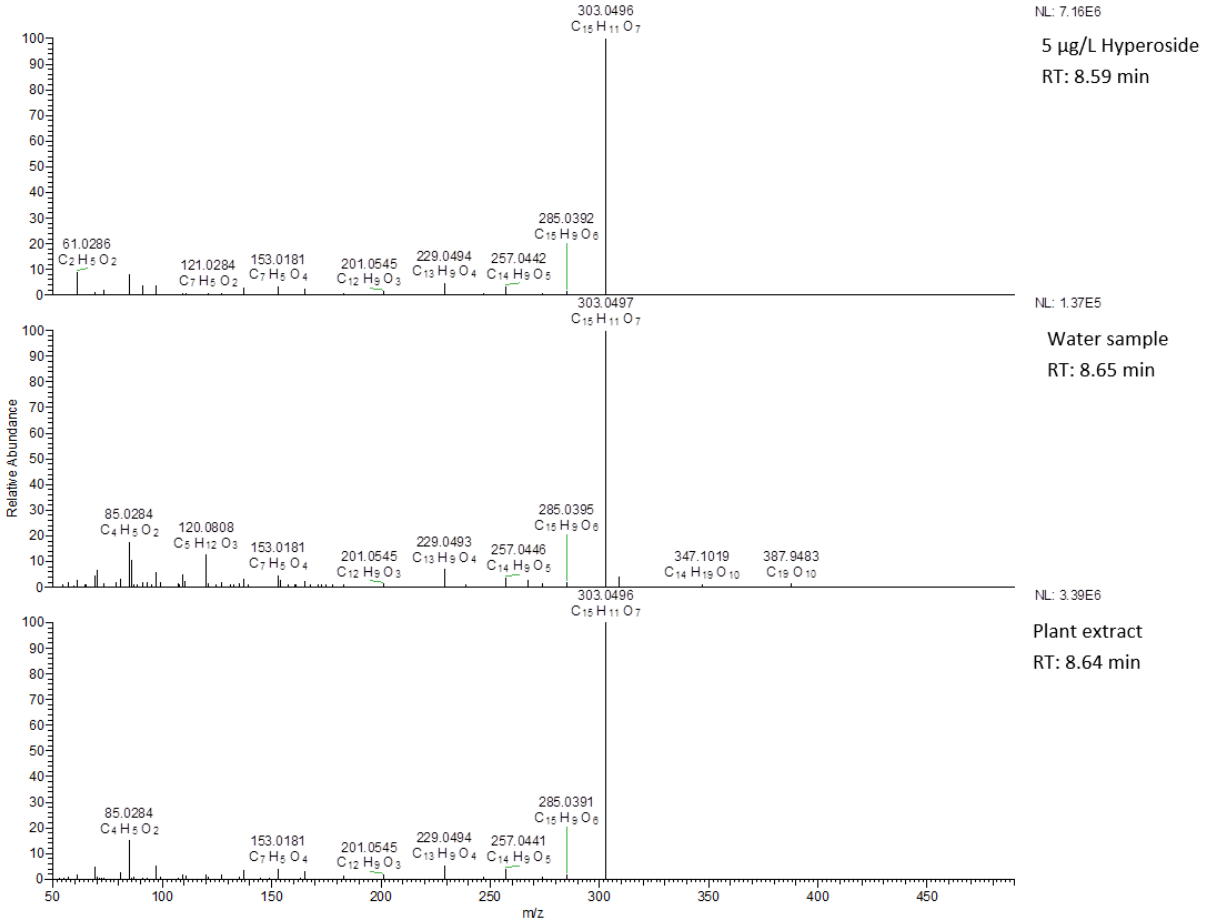

Figure S9: MS/MS spectra (HCD fragmentation at 55 a.u.) of hyperoside in a reference standard, water sample and plant (*Galanthus nivalis*) elutriates.

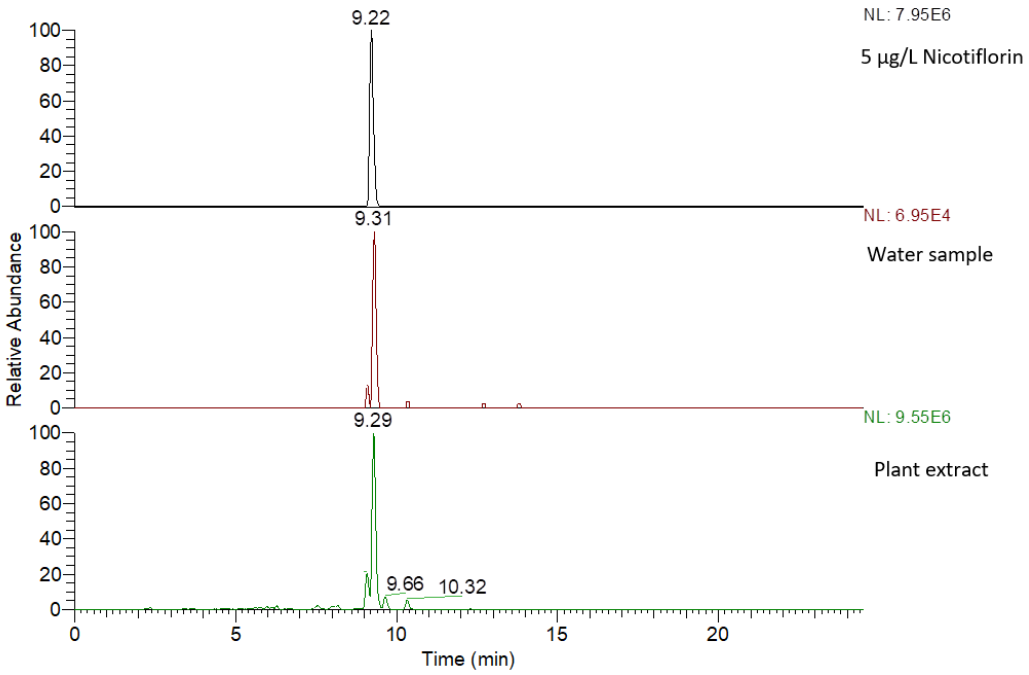

Figure S10: Extracted ion chromatograms of nicotiflorin in reference standard, water sample and plant (*Fraxinus excelsior*) elutriates. NL: signal intensity at 100%.

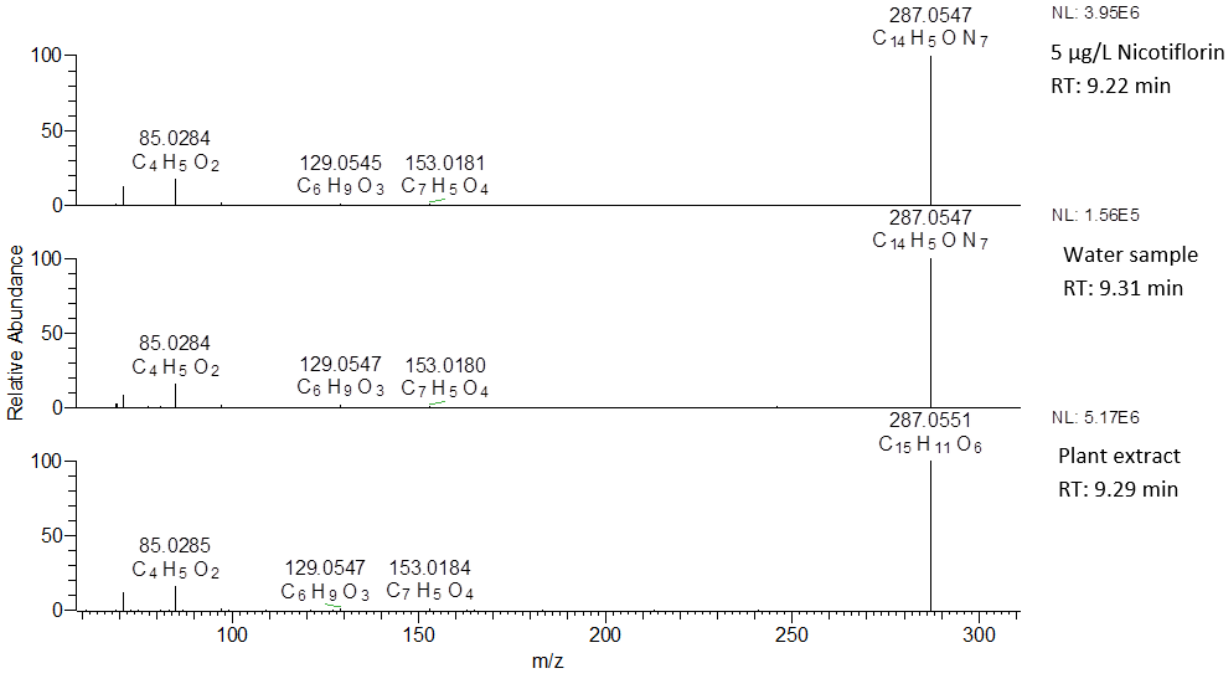

Figure S11: MS/MS spectra (HCD fragmentation at 45 a.u.) of nicotiflorin in a reference standard, water sample and plant (*Fraxinus excelsior*) elutriates.

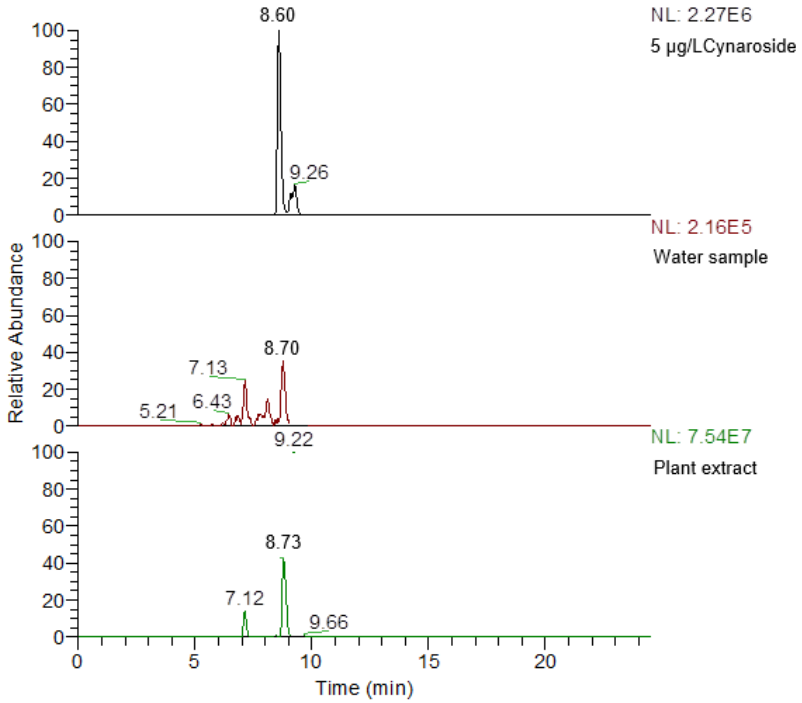

Figure S12: Extracted ion chromatograms of cynaroside in reference standard, water sample and plant (*Galanthus nivalis*) elutriates. NL: signal intensity at 100%.

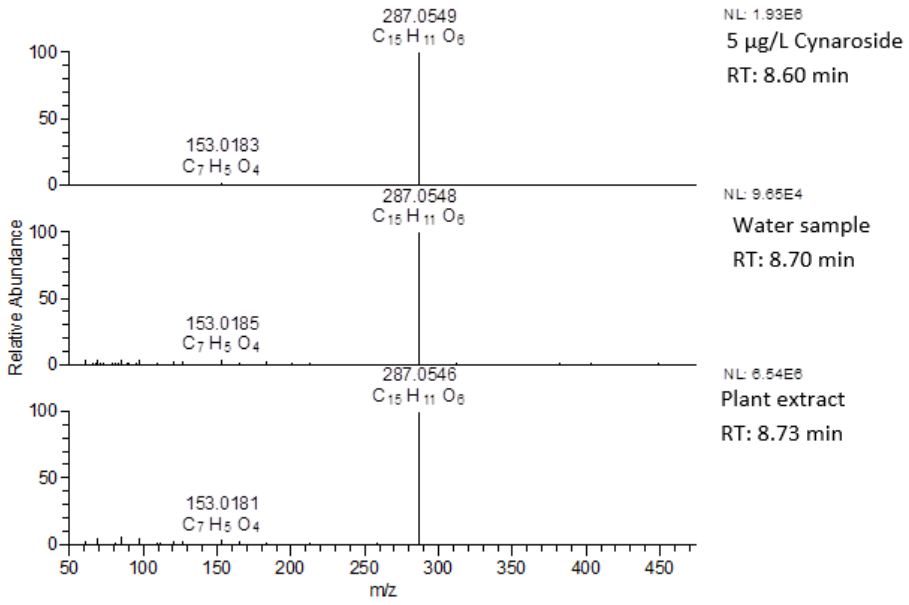

Figure S13: MS/MS spectra (HCD fragmentation at 45 a.u.) of cynaroside in a reference standard, water sample and plant (*Galanthus nivalis*) elutriates.

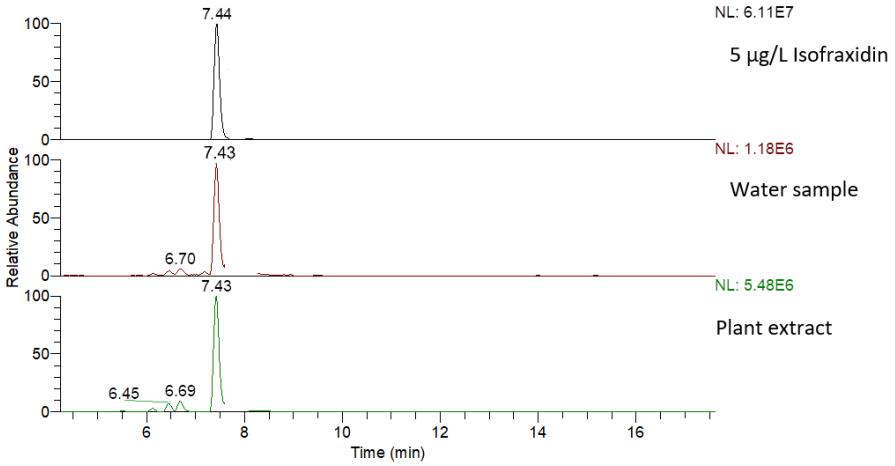

Figure S14: Extracted ion chromatograms of isofraxidin in reference standard, water sample and plant (*Fraxinus excelsior*) elutriates. NL: signal intensity at 100%.

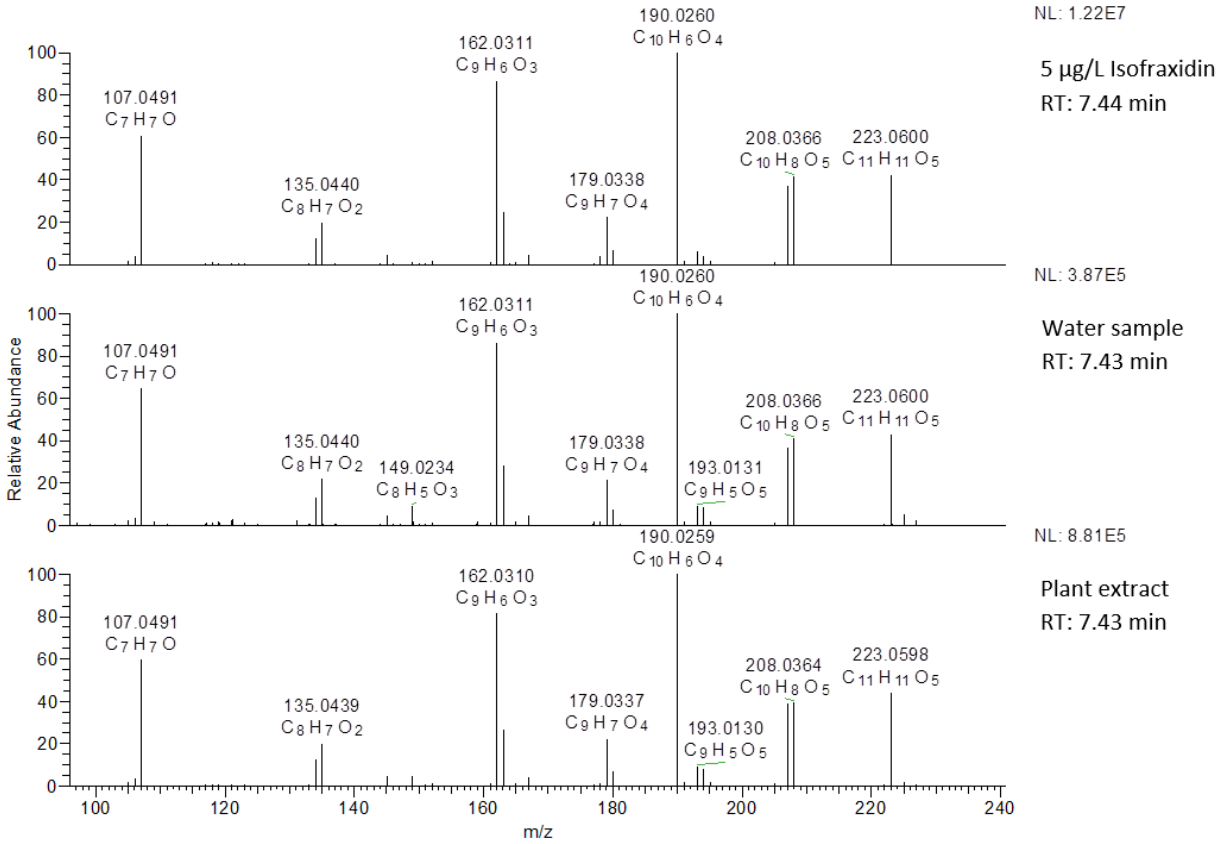

Figure S15: MS/MS spectra (HCD fragmentation at 55 a.u.) of isofraxidin in a reference standard, water sample and plant (*Fraxinus excelsior*) elutriates.

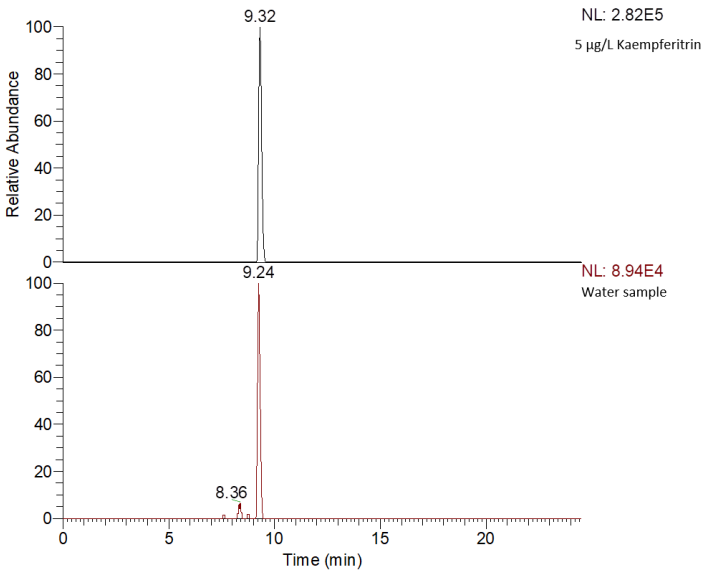

Figure S16: Extracted ion chromatograms of kaempferitrin in reference standard and water sample. NL: signal intensity at 100%.

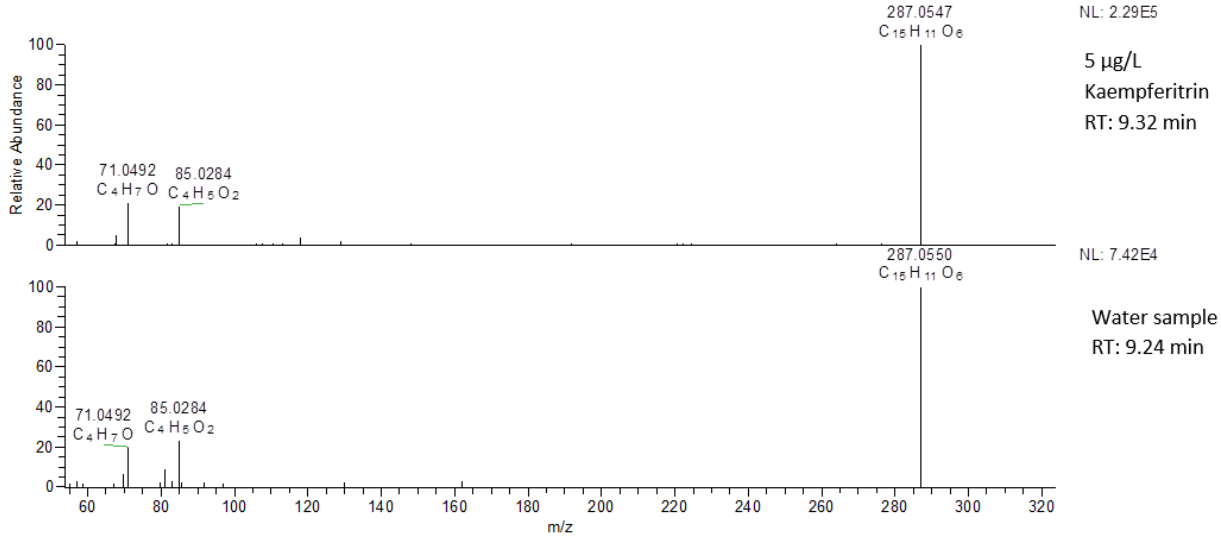

Figure S17: MS/MS spectra (HCD fragmentation at 45 a.u.) of kaempferitrin in a reference standard and water sample.

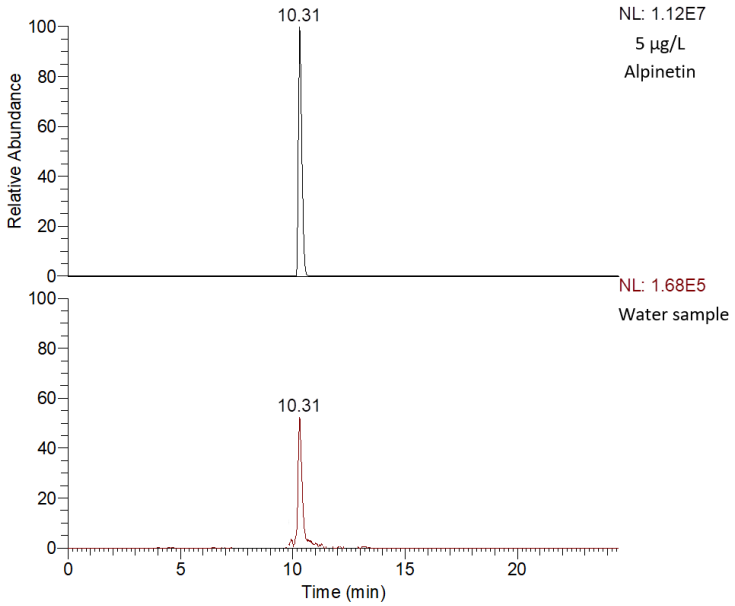

Figure S18: Extracted ion chromatograms of alpinetin in reference standard and water sample. NL: signal intensity at 100%.

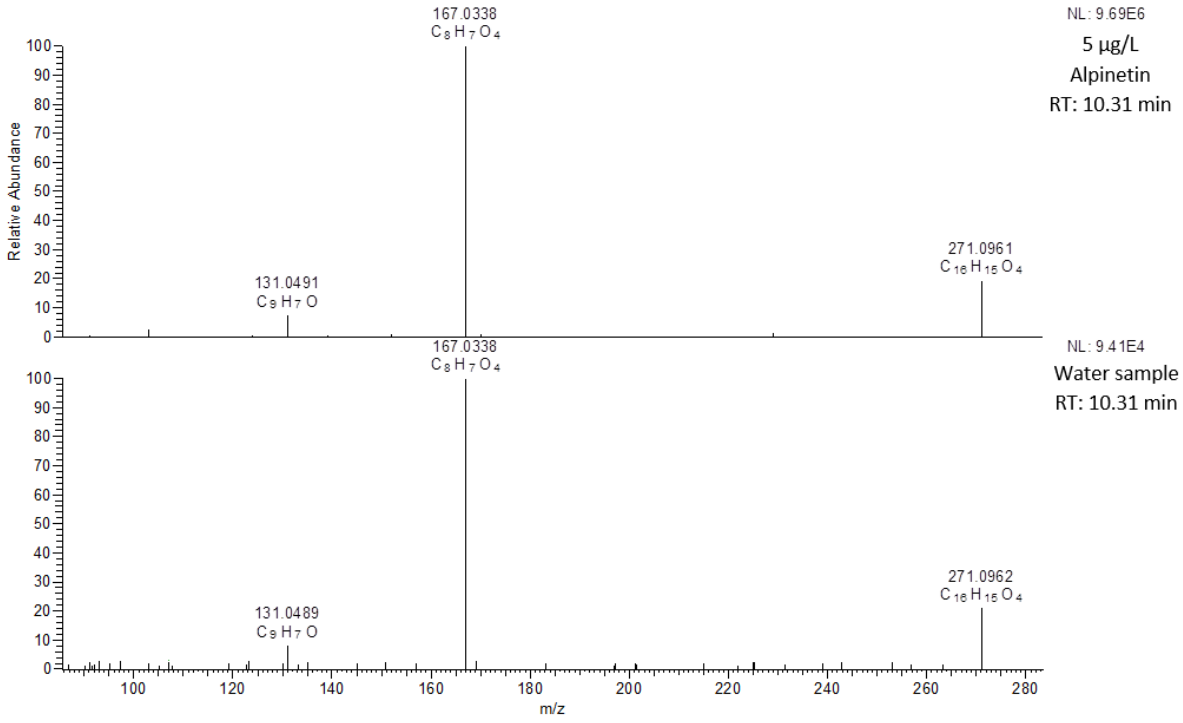

Figure S19: MS/MS spectra (HCD fragmentation at 45 a.u.) of alpinetin in a reference standard and water sample.

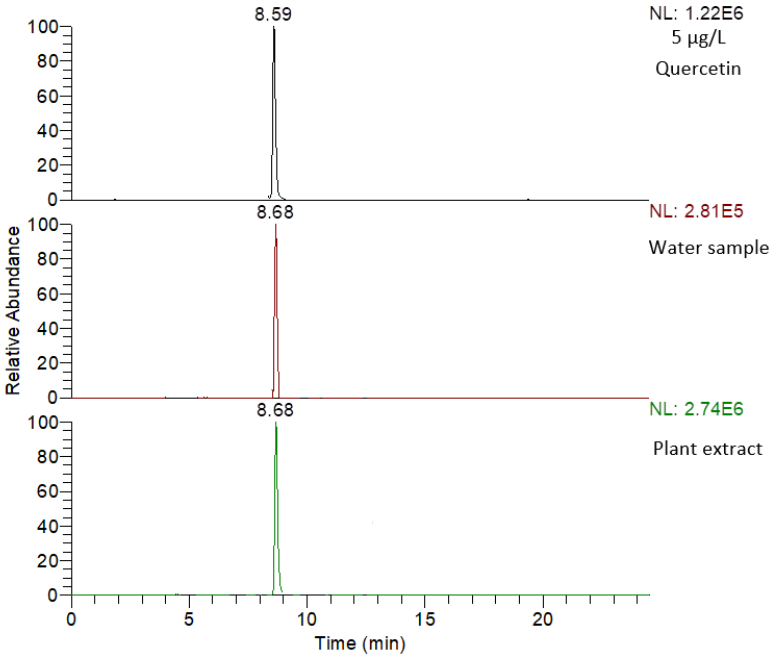

Figure S20: Extracted ion chromatograms of quercetin in reference standard, water sample and plant (*Fraxinus excelsior*) elutriates. NL: signal intensity at 100%.

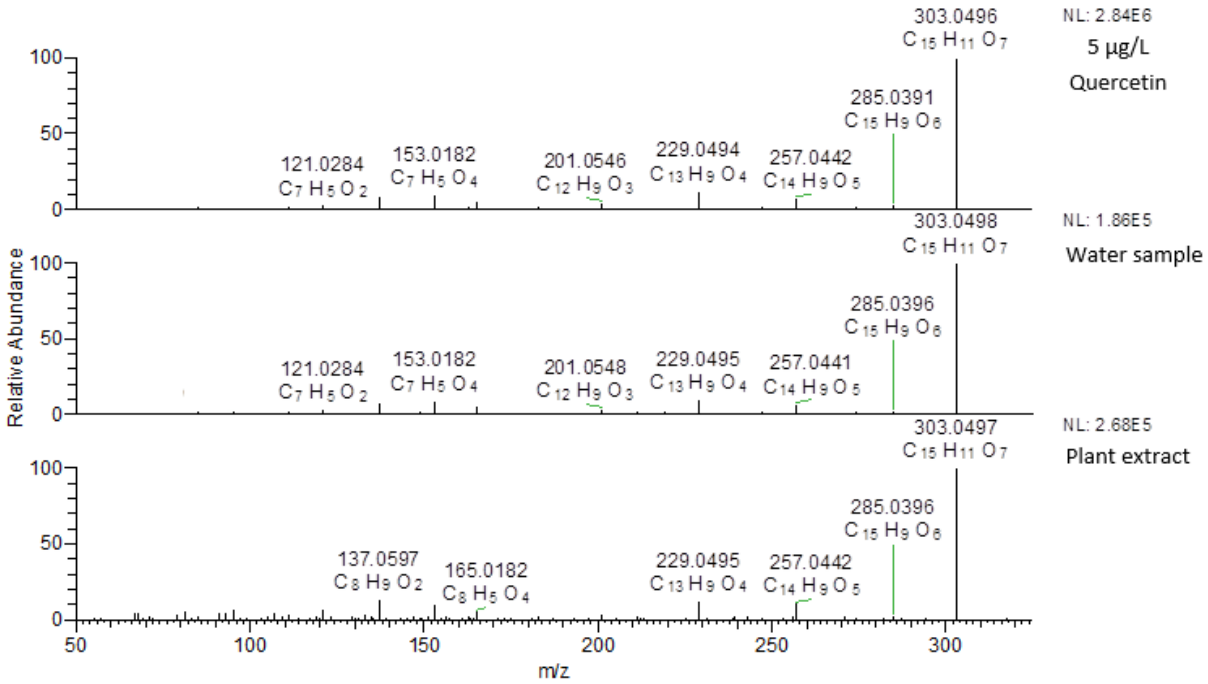

Figure S21: MS/MS spectra (HCD fragmentation at 55 a.u.) of quercetin in a reference standard, water sample and plant (*Fraxinus excelsior*) elutriates.

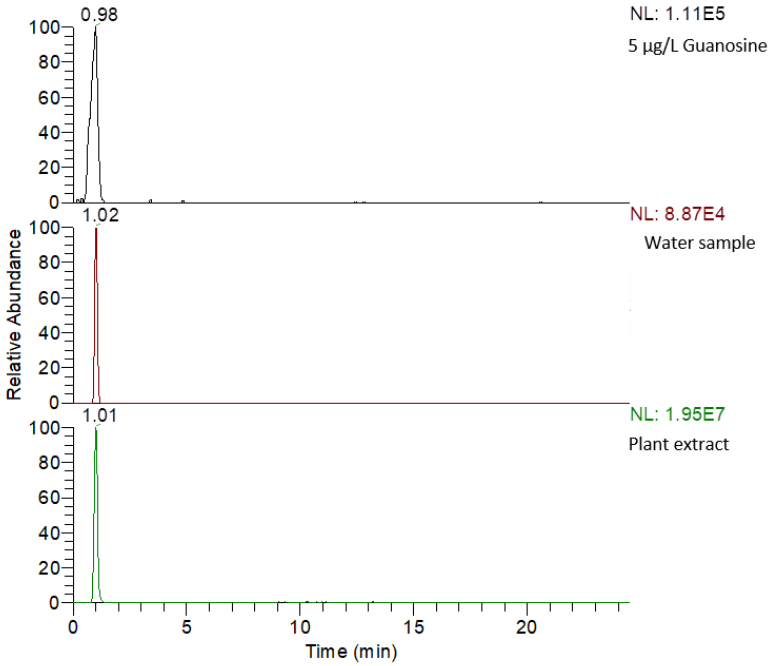

Figure S22: Extracted ion chromatograms of guanosine in reference standard, water sample and plant (*Digitalis purpurea*) elutriates. NL: signal intensity at 100%.

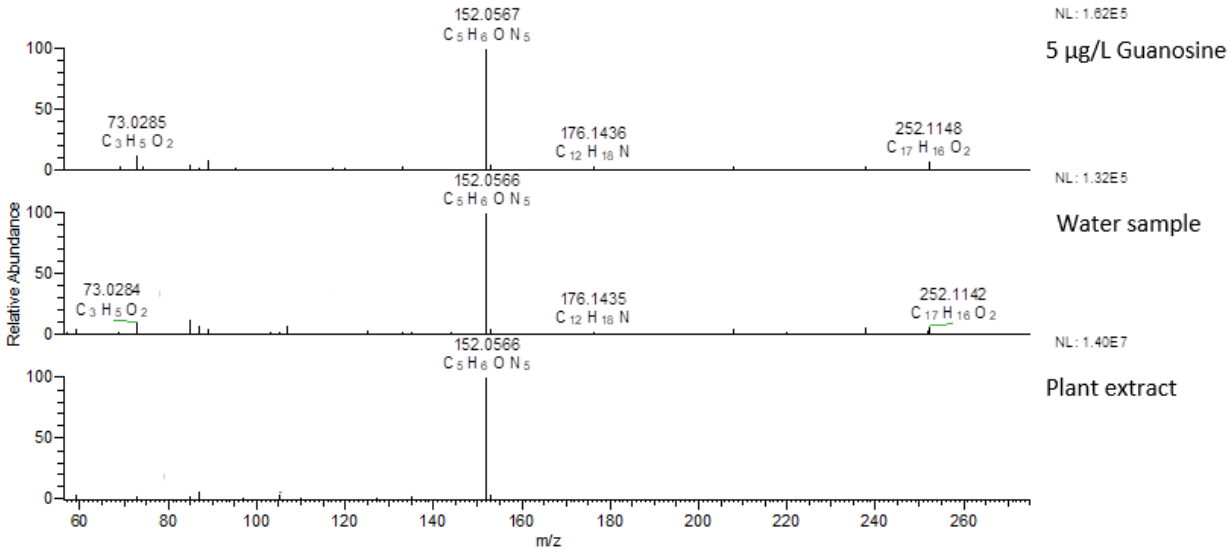

Figure S23: MS/MS spectra (HCD fragmentation at 45 a.u.) of guanosine in a reference standard, water sample and plant (*Digitalis purpurea*) elutriates.

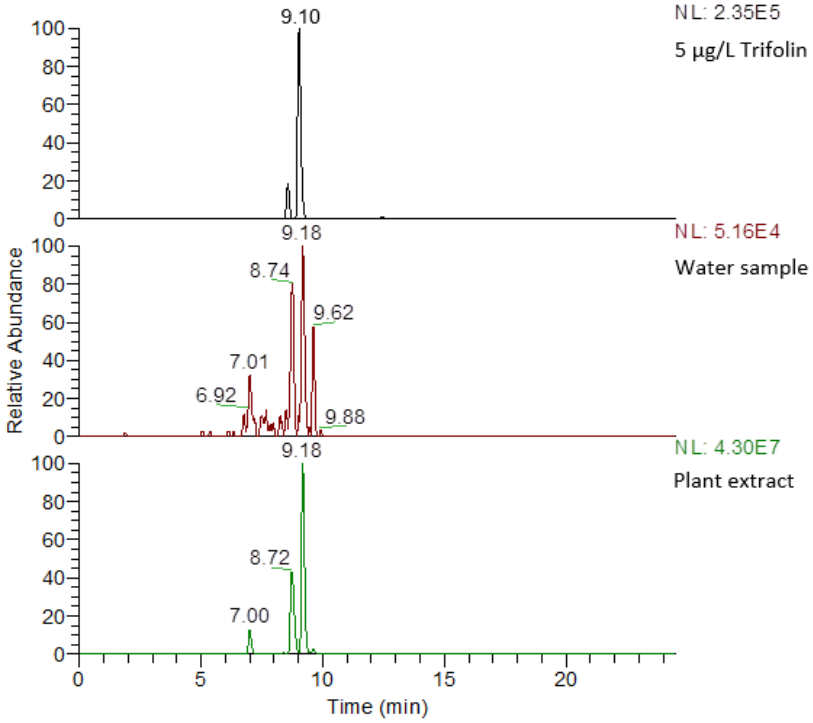

Figure S24: Extracted ion chromatograms of trifolin in reference standard, water sample and plant (*Galanthus nivalis*) elutriates. NL: signal intensity at 100%.

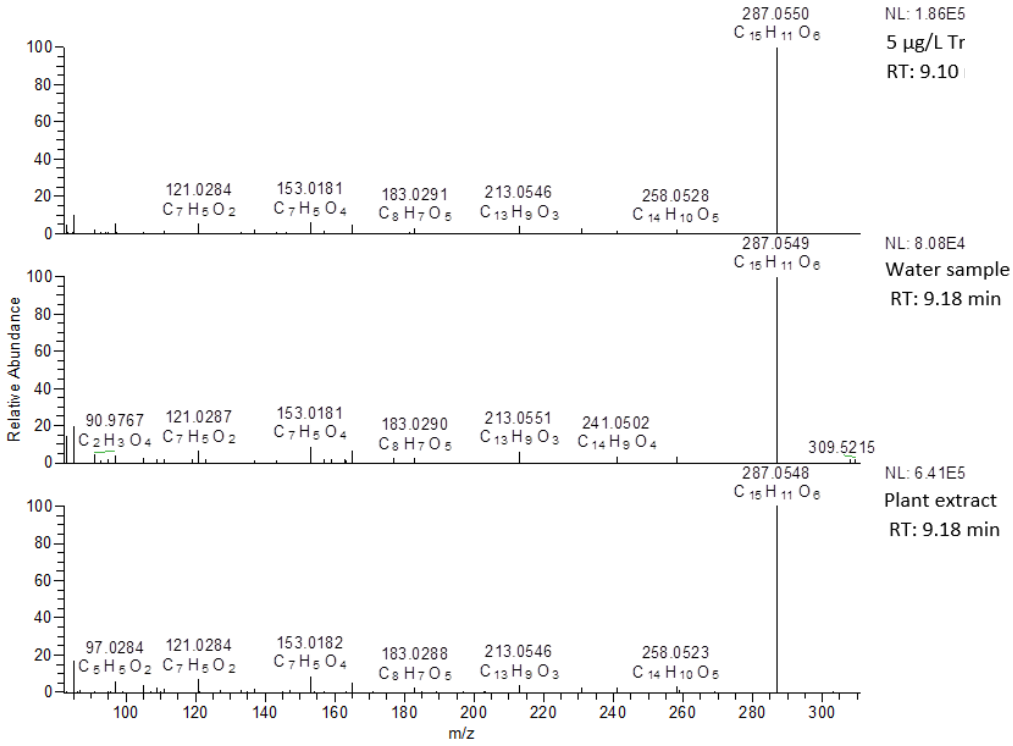

Figure S25: MS/MS spectra (HCD fragmentation at 45 a.u.) of trifolin in a reference standard, water sample and plant (*Galanthus nivalis*) elutriates.

Table S5: Concentration of detected metabolites in both water samples and aqueous plant elutriates.

Plant Metabolite	Concentration (µg/L) of metabolites detected in water samples								Water extractable concentration (µg/g) of metabolites in aqueous plant extract				
	Leipzig					Bode catchment			Leipzig		Bode catchment		
	Paußnitz-1 (ELP1)	Paußnitz-2 (ELP11)	Tributary to Elsterflutbett-1 (ELP2)	Tributary to Elsterflutbett-2 (ELP21)	Östliche Rietzchke (ELP3)	Barrenbach (BD1)	Getel (BD2)	Drangetalwasser (BD3)	*Allium ursanium*	*Galanthus nivalis*	*Fraxinus excisivor*	*Digitalis purpurea*	*Conium maculatum L*
Adenine	0.9	0.4	2.7	2.6	ND	1.5	0.9	2.4	47.8	9.2	59.3	47.5	35.0
Alpinetin	ND	0.02	ND	ND	0.05	ND	ND	ND	ND	ND	ND	ND	ND
Apiin	ND	ND	2.9	3.0	1.2	ND	ND	5.1	ND	ND	ND	21.7	ND
Cynaroside	0.4	ND	2.0	2.1	0.2	ND	ND	1.0	17.3	50.6	ND	11.1	ND
Guanosine	ND	ND	ND	3.9	ND	2.4	1.1	4.0	42.8	55.7	189.5	56.1	71.7
Hyperoside	ND	ND	4.0	3.8	ND	ND	ND	ND	ND	18.9	22.6	ND	ND
Isofraxidin	0.3	0.1	0.2	0.2	0.02	0.04	0.1	ND	0.1	0.01	16.8	0.1	0.04
Kaempferitrin	ND	ND	ND	ND	0.9	ND	ND	ND	ND	ND	ND	ND	ND
Nicotiflorin	ND	ND	2.2	2.2	ND	ND	1.9	ND	ND	ND	88.0	ND	ND
Quercetin	ND	ND	2.5	1.9	ND	ND	ND	ND	ND	74.7	54.6	ND	ND
Trifolin	ND	ND	2.0	2.9	ND	ND	0.3	ND	25.0	27.4	36.0	27.4	ND

ND – Not Detected

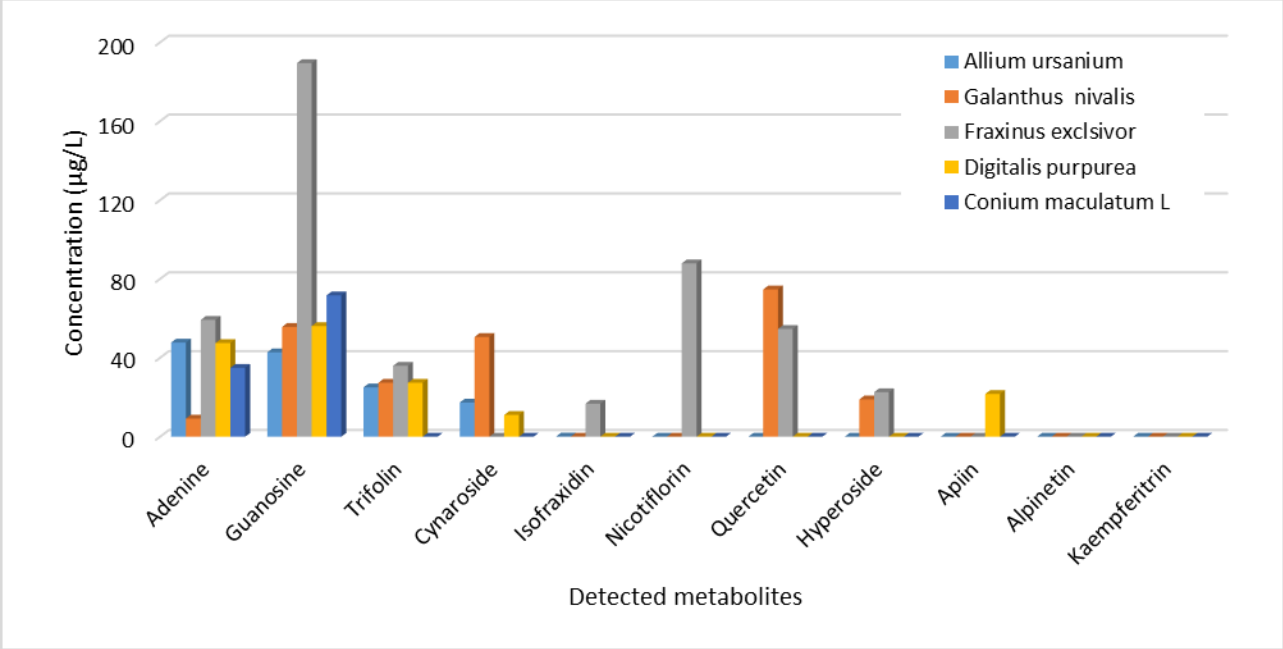

Figure S26: *Distribution of detected metabolites in the aqueous plant elutriates*

A.2 Target screening of plant secondary metabolites in river waters by liquid chromatography coupled to high-resolution mass spectrometry (LC–HRMS)

Nanusha MY, Krauss M, Schönsee CD, Günthardt BF, Bucheli TD and Brack W, 2020. Environ Sci Eur 32:142 (doi.org/10.1186/s12302-020-00399-2)

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What are the contributions of the doctoral candidate and his co-authors?

1. **Concept and design**
   - ✓ Doctoral candidate: 65 %
   - ✓ Co-author 6: 30 %
   - ✓ Co-author 2,3,4 and 5: 5 %
2. **Conducting tests and experiments**
   - ✓ Doctoral candidate: 90 %
   - ✓ Co-author 2,6: 10 %
3. **Compilation of data sets and figures**
   - ✓ Doctoral candidate: 85 %
   - ✓ Co-author 2,6: 15 %
4. **Analysis and interpretation of data**
   - ✓ Doctoral candidate: 85 %
   - ✓ Co-author 2,6: 15 %
5. **Drafting of manuscript**
   - ✓ Doctoral candidate: 80 %
   - ✓ Co-author 2,6: 15 %
   - ✓ Co-author 3, 4 and 5: 5%

I hereby certify that the information above is correct.

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Environmental Sciences Europe

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Target screening of plant secondary metabolites in river waters by liquid chromatography coupled to high-resolution mass spectrometry (LC–HRMS)

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Abstract

Background: Substantial efforts have been made to monitor potentially hazardous anthropogenic contaminants in surface waters while for plant secondary metabolites (PSMs) almost no data on occurrence in the water cycle are available. These metabolites enter river waters through various pathways such as leaching, surface run-off and rain sewers or input of litter from vegetation and might add to the biological activity of the chemical mixture. To reduce this data gap, we conducted a LC–HRMS target screening in river waters from two different catchments for 150 plant metabolites which were selected from a larger database considering their expected abundance in the vegetation, their potential mobility, persistence and toxicity in the water cycle and commercial availability of standards.

Results: The screening revealed the presence of 12 out of 150 possibly toxic PSMs including coumarins (bergapten, scopoletin, fraxidin, esculetin and psoralen), a flavonoid (formononetin) and alkaloids (lycorine and narciclasine). The compounds narciclasine and lycorine were detected at concentrations up to 3 µg/L while esculetin and fraxidin occurred at concentrations above 1 µg/L. Nine compounds occurred at concentrations above 0.1 µg/L, the Threshold for Toxicological Concern (TTC) for non-genotoxic and non-endocrine disrupting chemicals in drinking water.

Conclusions: Our study provides an overview of potentially biologically active PSMs in surface waters and recommends their consideration in monitoring and risk assessment of water resources. This is currently hampered by a lack of effect data including toxicity to aquatic organisms, endocrine disruption and genotoxicity and demands for involvement of these compounds in biotesting.

Keywords: Natural toxins, Bioactive compounds, Mixture toxicity, Surface water, Emerging contaminants

Background

Plants produce a large variety of chemical compounds, which may be categorized as primary and secondary metabolites. Primary metabolites are necessary for growth and maintenance of cellular functions of the plant while secondary metabolites play an important role, for example as defence (against herbivores, microbes, viruses or competing plants) and signal compounds to attract pollinating or seed dispersing animals [26, 36, 53]. Many PSMs can be seen as nature's own pesticides and have the potential to contribute to adverse effects of chemical mixtures in aquatic ecosystems together with anthropogenic chemicals [47]. Often, the production of PSMs is specific for taxonomic groups, species, genera or families. The amounts produced are typically lower than

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those of primary metabolites [36]. Secondary metabolites are diverse in their chemical nature. Most of them belong to four major classes of compounds, namely terpenoids, phenolic compounds, alkaloids and sulfur-containing compounds [13]. Due to their biological activity, PSMs have been used for drug development including anti-inflammatory, antioxidant and antiviral agents. However, many medicinally active PSMs also show toxic side effects [10, 11, 26, 39]. For instance, bergapten, a furanocoumarin, has shown antitumor effects in a variety of cell types, but is also a potential photosensitizer that can cause severe phytophotodermatitis after either skin contact or ingestion followed by sun UV exposure [9, 43, 51]. Some PSMs detected in the water cycle have been shown to cause severe impacts on human health such as aristolochic acids from *Aristolochia clematitis* causing Balkan endemic nephropathy [48] and the potent carcinogen ptaquiloside. The latter is produced by bracken fern and emitted into the water cycle particularly during rain events at toxicologically relevant concentrations [7, 35]. Recently, natural carboline alkaloids have been demonstrated to exhibit synergistic mutagenic effects with anthropogenic aromatic amines [31].

Plants produce toxic PSMs particularly under environmental stress and release these compounds to the environment through various means such as root exudates, volatilization and animal contact as part of their defence mechanism [2, 4, 10, 26]. Previous research demonstrated their pharmacological effect and toxicity by isolating them from plants [3, 42] and their contribution to mixture toxic risk in river water [5, 34]. In silico predictions suggest that many PSMs are persistent and mobile in the environment [14]. The authors identified priority phytotoxins characterized based on in silico-predicted values of half-life longer than 20 days, a log D_{OC} (organic carbon–water partition coefficient) below 4.5, rodent or aquatic toxicity and high abundance of the producing plant in Switzerland [14]. Assuming similar vegetation in Germany, these priority phytotoxins were used as a basis for target selection in the present study. PSMs may be transported to river water through leaching, rain sewers and surface run-off and might pose a risk not only to aquatic organisms, but also to human health in case of exposure, if the water is used for human consumption and recreational purposes. Recently, target and suspect screening of PSMs identified 12 compounds in Swiss small creeks from three compound classes including formononetin, an estrogenic isoflavone, the indole alkaloid gramine and several pyrrolizidine alkaloids [15]. Formononetin in concert with other isoflavones has been detected in Swiss and USA surface waters already earlier [16, 23]. Along with other organic matters the coumarins esculetin and umbelliferone were previously reported in Suwannee River fulvic acid isolates, USA [41]. Thus, PSMs may add to the complex mixtures of anthropogenic organic micropollutants in water resources. Therefore, there are indications that some PSMs, which exhibit toxicity at environmental concentrations, may jeopardize water quality and affect aquatic ecosystems and human health in concert with anthropogenic compounds.

The objective of the present study was to perform a first river water target screening of PSM selected from a larger database for their expected mobility, persistence and toxicity, their expected abundance and their commercial availability as standards in two selected catchments with primarily natural vegetation and agricultural land use, respectively. We focused particularly on sampling during or after rain events in the vegetation season to enhance the probability of detection of PSMs leaching to the river water. Since hardly any quantitative toxicity data for PSMs is available, we compared water concentrations with TTC suggested for drinking water contaminants for which no toxicity data exist for a preliminary estimate of risks [29].

Experimental section

Study site and water sampling

The investigated catchments are located in the northwest part of the federal state of Saxony (close to the city of Leipzig) and in Saxony-Anhalt (Bode catchment), Germany. Both catchments were selected due to their land coverage with natural and/or agricultural vegetation along the river banks. The 50 km^2 large floodplain forest in Leipzig reaches along the rivers Elster, Pleiße and Luppe (EPL catchment) together with some smaller tributaries. The natural old-growth deciduous forest was historically used for the extraction of coppice and clay. It is mainly characterized by *Quercus robur*, *Fraxinus excelsior*, *Acer pseudoplatanus*, *Ulmus minor*, *Alnus glutinosa*, *Tilia cordata*, *Carpinus betulus*, *Acer platanoides* and *Acer campestre*. In spring, the forest scrub is dominated by monocotyledonous and tuberous plants such as *Allium ursinum*, *Anemone nemorosa*, *Galanthus nivalis* and *Leucojum vernum* [22]. Thus, during their periodic growth and decay, they might leave their secondary metabolite footprints in the environmental components (e.g., soil, river). The Bode catchment is characterized by large diversity of natural and agricultural vegetation. Land use is dominated by forest (such as broad-leaved forest, coniferous forest and mixed forest) in the mountain areas and agriculture in the lowland areas. Main crops include winter wheat, triticale, winter barley, rye, rape, sugar beet and corn.

Grab water samples were collected in the vegetation growing season of the years 2018 and 2019 in spring and summer during and after rain events when leaching of

PSMs was expected. A total of 38 rain event river samples were collected from both locations—23 samples from 6 streams situated in Leipzig and 15 samples from 15 streams in the Bode catchment (Fig. 1). In the ELP catchment, samples were taken repeatedly from the same spot at different rain event days. These samples were complemented with 18 dry weather samples (8 and 10 samples from ELP and Bode catchments, respectively) from different seasons for comparison (for more information on samples see Table S1 in supplementary information (Additional file 1). Water samples were taken with precleared glass beaker (500 mL) and solids were allowed to settle for about 2 min before transferring to sampling bottle. Aliquots of 1 mL were transferred to 2-mL autosampler vials for the chemical analysis. To minimize the interferences, all sampling bottles and laboratory vessels

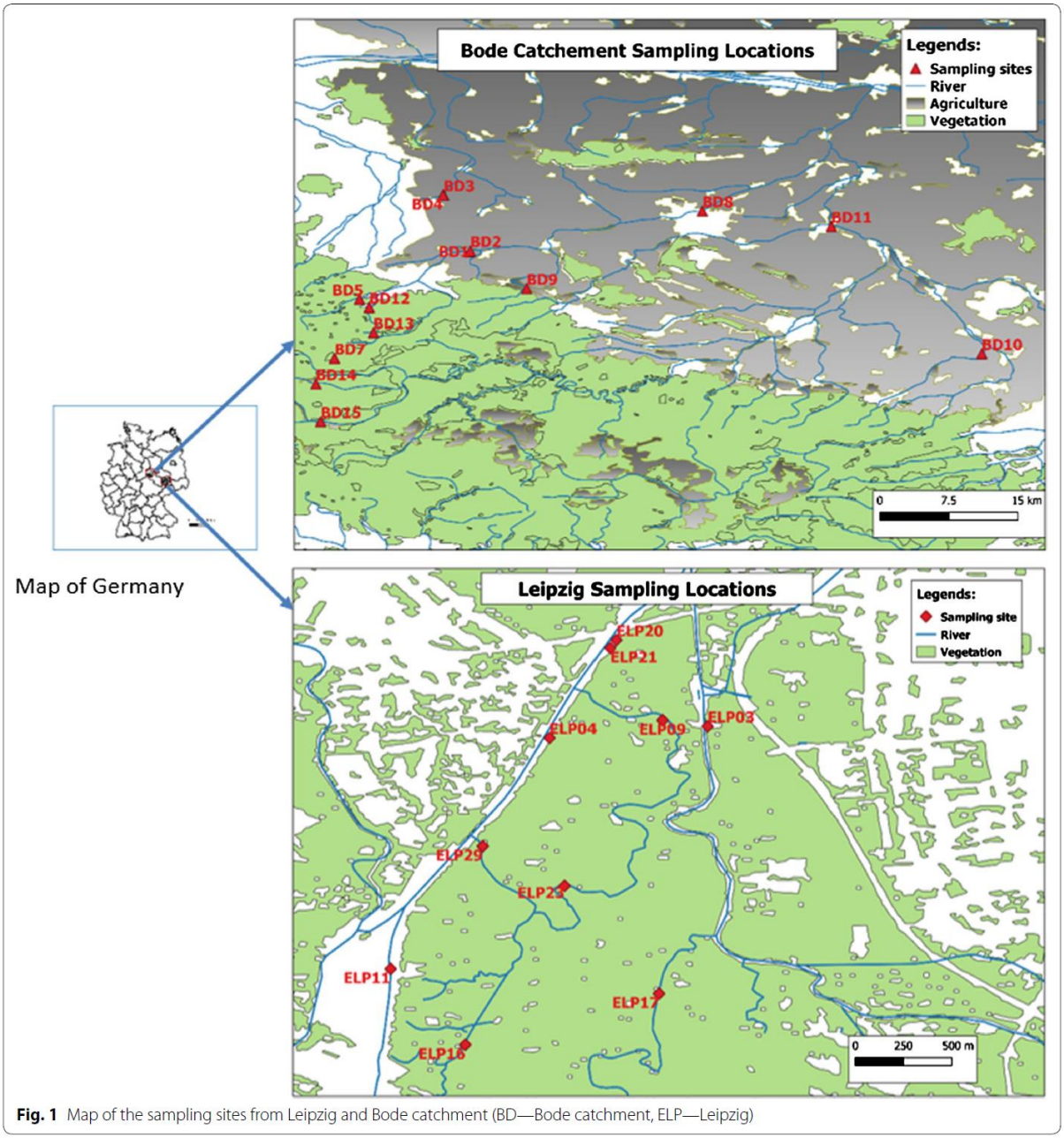

Fig. 1 Map of the sampling sites from Leipzig and Bode catchment (BD—Bode catchment, ELP—Leipzig)

were washed and rinsed with ethyl acetate, acetone and methanol before use. Field trip and laboratory blanks were also included to control interferences during the sampling campaign and transportation. Samples were chilled with ice packs during transportation to the UFZ laboratory, and then stored at − 24 °C until analysis.

Target secondary metabolite selection

Due to limited information on PSM, in silico evaluations were performed to assess their likelihood to occur in water. Prioritization for target screening was built on previous work by Gunthardt et al., identifying plant toxins with a high probability to reach the aqueous environment due to mobility and persistence [14]. The selected metabolites represent structurally diverse natural compounds from plant species. Thus, taking these PSM as the basic population of candidate compounds, we produced a shortlist of 150 metabolites also considering commercial availability and the probability of occurrence due to the abundance of the plants identified as the origin of these metabolites (for more information see Additional file 1: Table S2). Furthermore, only metabolites containing one or more of the elements nitrogen, oxygen and sulfur, in addition to carbon and hydrogen were selected to allow for a likely ionization by an electrospray ion source [30].

Chemical analysis

Water samples containing suspended matter were filtered using a glass fiber filter (Whatman GF/A, diameter 47 mm). Samples were prepared for direct injection by adding 25 µL of an internal standard mixture (40 ng/L), 25 µL of methanol (LC–MS grade) and 10 µL of ammonium formate buffer (2 M, pH = 3.5) to each 1-mL sample aliquot (see Additional file 1: Table S4 for more information on internal standards). For the chemical analysis, 100 µL of the sample was injected into a Thermo Ultimate 3000 LC system (consisting of a ternary pump, autosampler and column oven) coupled to a quadrupole-orbitrap instrument (Thermo QExactive Plus) equipped with a heated electrospray ionization (ESI) source.

Liquid chromatography

LC separation was performed on a Kinetex C18 EVO column (50 × 2.1 mm, 2.6 µm particle size) using a gradient elution with 0.1% of formic acid (eluent A) and methanol containing 0.1% of formic acid (eluent B) at a flow rate of 300 µL/min. After 1 min elution with 5% B, the fraction of B was linearly increased to 100% within 12 min and 100% B were kept for 11 min. Subsequently, the column was rinsed with a mixture of isopropanol + acetone 50:50/eluent B/eluent A (85%/10%/5%) to remove hydrophobic matrix constituents from the column. Finally, the column was re-equilibrated to initial conditions for 5.7 min. The column was operated at 40 °C.

Mass spectrometry

The heated ESI source and the transfer capillary were both operated at 300 °C, with a spray voltage of 3.8 kV (pos. mode), a sheath gas flow rate of 45 a.u. and an auxiliary gas flow rate of 1 a.u. The full-scan MS1 was recorded in an *m/z* range from 100 to 1500 with a nominal resolving power of 140,000 (referenced to *m/z* 200). For metabolite confirmation, data-dependent MS/MS acquisition was performed at a resolving power of 70,000 in additional runs (see Additional file 2: Table S5 for more information on MS setting). The MS was calibrated externally every 2 days using the calibration mixtures of the vendor, the mass accuracy was always below 5 ppm for all analyses. All MS analyses were performed in ESI positive mode (ESIpos) since we expected a better ionizability of SPMs than in/with ESI negative mode.

Target screening

Qualitative target screening

The LC–HRMS raw data were converted to mzML format using ProteoWizard (version 2.1.0) [17]. The centroid data were subjected to MZmine (version 2.38) for peak detection followed by peak alignment and identification (target compound annotation) [20, 21, 38]. Settings for each step of the data processing are given in Additional file 2: Table S3. Further evaluation and visualization were performed using Excel 2013 (Microsoft office) and R (version 3.4.3).

Targets were identified by matching *m/z* and retention time between water samples and standard compounds with a mass and retention time tolerance of 5 ppm and ± 0.1 min, respectively. Prior to clearing of false positives from the annotated list, the cut-off intensity was set to 10^4 to exclude signals due to noise and background. For blank correction, seven blanks were analyzed together with the samples to remove noise and background contaminants. Duplicates resulting from multiple annotation were removed manually using peak resolution and intensity (for detailed steps on workflow see Additional file 2: Figure S1). For the tentatively identified target compounds, an inclusion list was developed for data-dependent acquisition (MS/MS). MS/MS experiments were conducted on authentic standard compounds and the samples to confirm the chemical structure. Diagnostic MS/MS fragments were matched with the MS/MS of reference standards. For the target compounds with low intensity in unresolved chromatograms, parallel reaction monitoring analysis was conducted for better chromatographic peaks visualization. The XCalibur v4.0.27.10 (Thermo Fisher Scientific) software was used

for analysis of extracted ion chromatograms (EICs) and mass spectra (MS1 and MS2).

Quantification of detected metabolites

TraceFinder 3.2 (ThermoFisher Scientific) was used for the quantification of the 12 confirmed target PSMs using extracted ion chromatograms of the full-scan data. In TraceFinder, the use of only one identifier mass (precursor ion) bares the risk of false-positive identification and quantification of contaminants. Thus, additional fragment ions were used to confirm the presence of target compounds and to eliminate errors in identification (see Additional file 2: Table S6). For some metabolites, ions used for confirmation were not clearly detectable due to low intensity. In such cases, confirmation was complemented using Xcalibur. A series of calibration standards ranging from 1 to 5000 ng/L were used. All the calibration standards were treated exactly the same way as river water samples. The target compounds were quantified using the internal standards with the nearest retention time. The method detection limit (MDLs) (Table 1) for the detected PSMs were determined following US-EPA procedure [49]. The calculated concentrations below the MDLs were excluded.

Risk estimates

Due to a lack of toxicity data for our target compounds, we based a tentative risk estimate on TTC for non-genotoxic and non-endocrine disrupting compounds of 0.1 µg/L. We defined the ratio between measured concentration of the compounds i (c_i) and TTC as risk quotient (RQ), and calculated mixture RQs as the sum of individual RQs (Eq. 1) assuming a mixture RQ below one as safe for exposed humans and aquatic organisms:

$$\left(\sum \mathrm{RQ}\right) = \left(\sum c_i \mathrm{TTC}\right) \quad (1)$$

Results and discussion

Metabolites detected in river waters

Peak picking followed by annotation (based on MS1 full-scan) resulted in 145 target peaks with *m/z* and retention time matching to the target metabolite with a tolerance of 5 ppm and ± 0.1 min, respectively. Some target metabolites were annotated multiple times due to picking multiple peaks at a single precursor ion mass with given retention time tolerance. Removal of false positives and peak filtering using intensity and resolution reduced the target list to 106 peaks. Based on additional MS/MS fragment comparison with reference standards, we confirmed the presence of 12 target metabolites in the river waters (see Additional file 2: Figure S2–S13 for MS spectra). For the rest (94), MS/MS fragment did not match between water sample and their respective reference standard, thus discarded. They could be isobaric compounds, annotated in the given retention time window. The identified compounds belong to different classes of natural compounds including coumarins, alkaloids, isoflavone and others. In general, the identified metabolites contain one or more phenolic groups representing a class of compounds found most abundantly in vegetation [1, 39]. The names and chemical structures of the identified metabolites are given in Fig. 2.

Table 1 The concentration range (min–max, ng/L) of identified plant metabolites in river water

Plant secondary metabolite	Chemical formula	CAS no.	*m/z* ($M+H^+$)	Retention time (min)	MDL (ng/L)	Concentration range (min–max, ng/L)		Frequency of detection	
						Leipzig	Bode	Leipzig	Bode
Coumarin	$C_9H_6O_2$	91-64-5	147.0441	7.3	11	12	43	1	1
Esculetin	$C_9H_6O_4$	305-01-1	179.0336	4.2	50	116–1658	104–157	2	3
Fraxidin	$C_{11}H_{10}O_5$	525-21-3	223.0600	7.8	4	56–1145	19–155	9	8
Scopoletin	$C_{10}H_8O_4$	92-61-5	193.0496	7.1	2	9–47	7–49	9	9
Bergapten	$C_{12}H_8O_4$	484-20-8	217.0495	10.1	4	510	541	1	1
Psoralen	$C_{11}H_6O_3$	66-97-7	187.0388	9.1	3	ND	141–224	0	2
Lycorine	$C_{16}H_{17}NO_4$	476-28-8	288.1225	1.0	3	1015–2331	11	2	1
Narciclasine	$C_{14}H_{13}NO_7$	29477-83-6	308.0765	5.7	150	507–3353	ND	2	–
Nicotine	$C_{10}H_{14}N_2$	54-11-5	163.1228	0.9	1.6	2–6	4–35	2	2
Piperine	$C_{17}H_{19}NO_3$	94-62-2	286.1434	11.9	0.9	1–338	4–294	4	2
Formononetin	$C_{16}H_{12}O_4$	485-72-3	269.0804	10.8	3	8–35	123	5	1
Coniferyl aldehyde	$C_{10}H_{10}O_3$	458-36-6	179.0701	7.6	8	13–46	ND	2	–

ND not detected

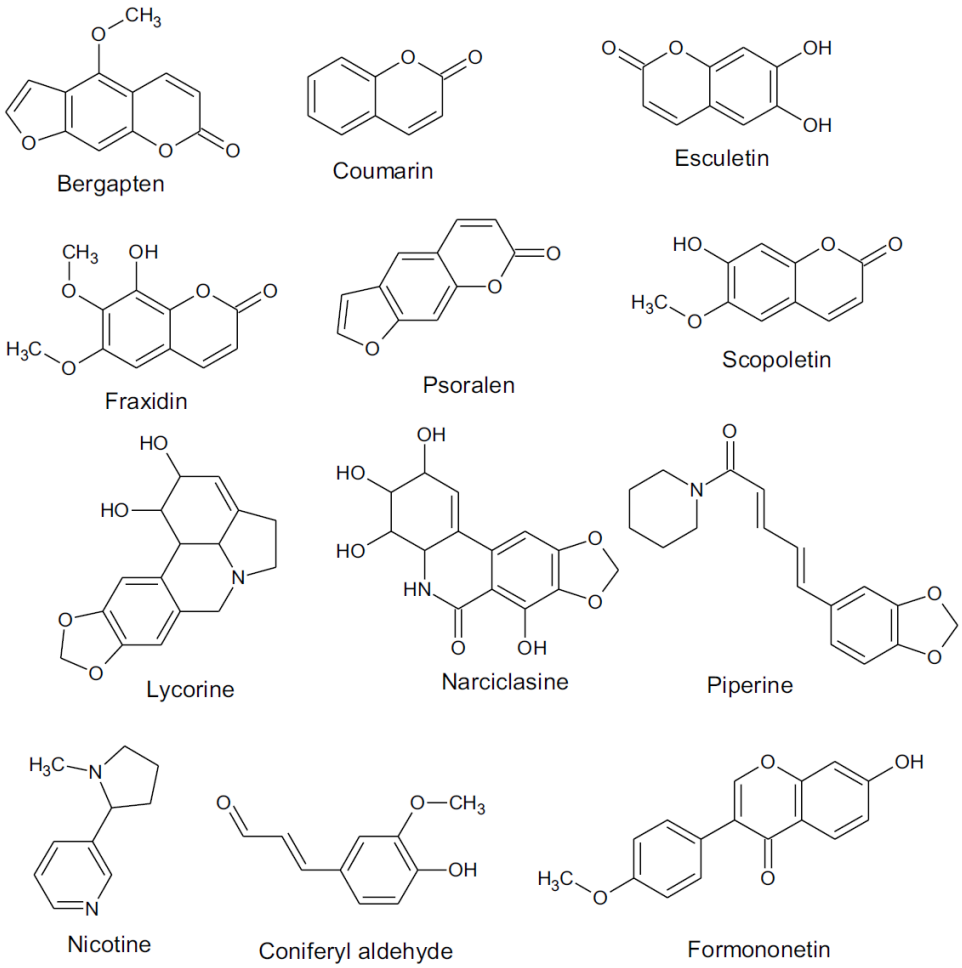

Fig. 2 Plant secondary metabolites identified in river waters

Distribution of measured metabolites in river waters

In 18 out of 38 rain event samples PSMs were detected (9 samples from each catchment—Leipzig and Bode), while in none of the dry weather control samples any of the target PSMs were found. It is apparent that, in about 50% (20) rain event samples the target compounds were not detected. In the Bode catchment, larger numbers of metabolites were detected in rivers impacted by agricultural than natural vegetation (Fig. 1 and Additional file 2: Table S7).

Among the identified 12 metabolites, 11 were detected in samples from the ELP catchment and 10 were found in the Bode catchment. In both catchments, the coumarin derivatives scopoletin and fraxidin were detected with the highest frequency with 9 samples from each catchments (Fig. 3a and b). Esculetin, another coumarin derivative was the third most frequently detected PSM in the Bode catchment with 20% while it was detected in 9% of the samples from the ELP catchment. The high detection frequency of fraxidin and esculetin is in good agreement with its formation by *Fraxinus excelsior*, a frequent tree in central European floodplains including the ones under investigation here. Scopoletin is produced by *Scopolia* species, but also the very frequently occurring stinging nettle *Urtica dioica*. However, all three compounds are present in a wide range of plants, which might contribute to emissions [52]. The isoflavone formononetin is the third most frequently occurring PSM in the ELP catchment with 22%, while it was found in 7% of the Bode catchment samples. Other compounds were detected only in specific samples from a specific season such as lycorine and narciclasine occurring in Amaryllidaceae, which show a high abundance within short

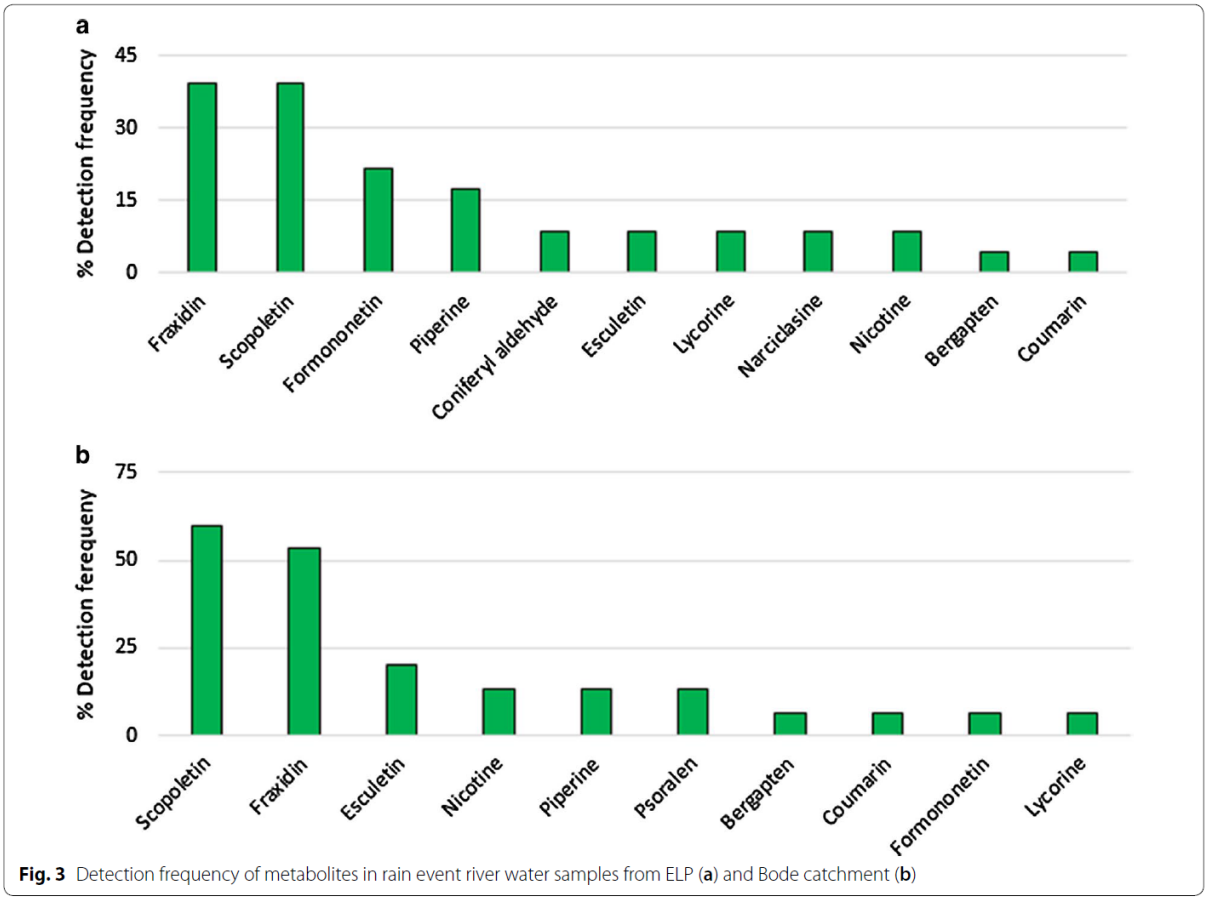

Fig. 3 Detection frequency of metabolites in rain event river water samples from ELP (**a**) and Bode catchment (**b**)

growth periods such as Galanthus species in early spring. Although coniferyl aldehyde is a lignin component of many plants, it could be detected only in the EPL catchment and the coumarin psoralen only in Bode catchment. Natural compounds stemming likely from human consumption such as the piperidine alkaloid piperine as a component of pepper and nicotine from tobacco could be detected in both catchments.

Quantification of PSMs in river water

The target PSMs were detected in a concentration range of 1–3400 ng/L (Table 1). The concentrations of identified metabolites in individual samples are given in Additional file 2: Table S7. The highest concentrations were detected for lycorine and narciclasine with maximum concentrations of 2 and 3 µg/L during the times of high abundance of *Galanthus* sp. (snowdrop) and *Leucojum vernum* (spring snowflake). The concentrations of these phytotoxins strongly exceed the TTC of 0.1 µg/L for non-genotoxic and non-endocrine disrupting compounds and would be of concern, if these water resources would be used for drinking water production. Both compounds are highly bioactive and toxic causing among others nausea and emesis in human and animals [24, 25]. Lycorine demonstrated acetylcholinesterase inhibition effects at IC_{50} of 213 µg/L [18, 32], while other authors reported above 1000 [6]. So, apart from its toxicity, lycorine also has more positive effects, as many SPMs, such as antibacterial, anti-viral, anti-malarial, anti-allergy effects, inhibits protein and DNA synthesis and has cardiovascular protection and antitumor effects [19].

The second group of PSMs exceeding the TTC of 0.1 µg/L is the coumarin derivatives with fraxidin and esculetin concentrations of 19 to 1145 ng/L and 116 to 1658 ng/L, respectively, while coumarin and scopoletin remained below 50 ng/L (Table 1). A maximum concentration of 300 µg/L esculetin was previously reported in Suwannee River fulvic acid isolates, USA [41]. In general, samples from the ELP catchment showed higher concentrations of coumarins than those from the Bode catchment. All four compounds have been isolated from *Fraxinus excelsior* [22, 40, 50, 52], a characteristic tree

along the rivers in both catchments. Coumarins comprise a very large class of substances, found in several higher plants and constitute fused benzene and pyrone rings [33, 45, 52]. Simple coumarins have been found to be biologically active with anti-stress, anti-fatigue, anti-gastric ulcer, anti-depressive, immuno-enhancing and anti-inflammatory effects [52, 54]. Scopoletin, isolated from *Scopolia carniolica* (Solanaceae), was shown to inhibit acetylcholinesterase at IC_{50} of 169 µg/L in vitro assay [18].

Two furanocoumarins, bergapten and psoralen, have been detected in only two samples, each, from both catchments but in all cases above the TTC with 510 and 541 ng/L for bergapten and 141 and 224 ng/L for psoralen. They are synthesized by several plants, especially by those of the Apiaceace family [43]. They are generally known for their strong photosensitizing activity when applied topically or accidentally get in contact to the skin. The exposure to furanocoumarins combined with long UV radiation causes cytotoxic reactions (e.g., erythema) and genotoxic responses by binding to nucleobases in DNA [43, 51].

The flavonoid, formononetin was detected in five samples from the ELP catchment at a concentration range of 8–35 ng/L and in one sample from Bode catchment with 123 ng/L again exceeding the TTC. The latter was taken from an agricultural area [16, 23]. The PSM occurs in many leguminous plants such as clover (*Trifolium*), an abundant species in fertile meadows and pastures but also beans such as green beans, lima beans and soy [1, 33, 39]. Formononetin has been shown to display estrogenic properties with an IC_{50} of 104 µg/L in vitro [28] and induce angiogenesis activities [27].

In both catchments, also the PSMs nicotine and piperine have been found in concentrations of 2 to 35 ng/L and 1–338 ng/L again with two samples exceeding the TTC. The input of both metabolites to the river water is very likely due to human activities, while no plants containing these compounds in the catchments are known. Both PSMs are widely consumed by humans and related to tobacco smoking and food flavoring, respectively. Nicotine is highly addictive and acts as a receptor agonist at most nicotinic acetylcholine receptors (nAChRs) [12]. Piperine is a major component of Piper species (e.g., *Piper nigrum*, *Piper longum*, *Piper officinarum* and *Piper retrofractum*), which are globally marketed as flavoring agent and cooking spice with a long history of human health benefits and a wide consumption [44, 46]. Piperine has been found to have numerous medicinal applications such as antioxidant, antiplatelet, anti-inflammatory, antihypertensive, hepatoprotective, antithyroid, antitumor, antiasthmatic activity and has also been used as fertility enhancer [8]. Apart from its numerous benefits, it may also have adverse effects including hemorrhagic necrosis and edema in gastrointestinal tract, urinary bladder and adrenal glands observed in animal tests with rats [37]. Zwart et al. detected piperine in waste water treatment plant effluent and classified it as one of the most potent nonsteroidal estrogens at EC_{50} of 300 ng/L in vitro [55], which is in the same order of magnitude as the concentrations obtained in the present study.

Co-occurrence of PSMs

Similar to anthropogenic compounds, also PSMs occur in mixtures. In all of the samples, where we detected our target PSMs, we found at least two of them, at two sites (11% (2) of positive samples), we detected even six co-occurring PSMs (Fig. 4a). The compounds fraxidin and scopoletin were common to all samples, with only one exception in the Bode catchment. Based on TTC of

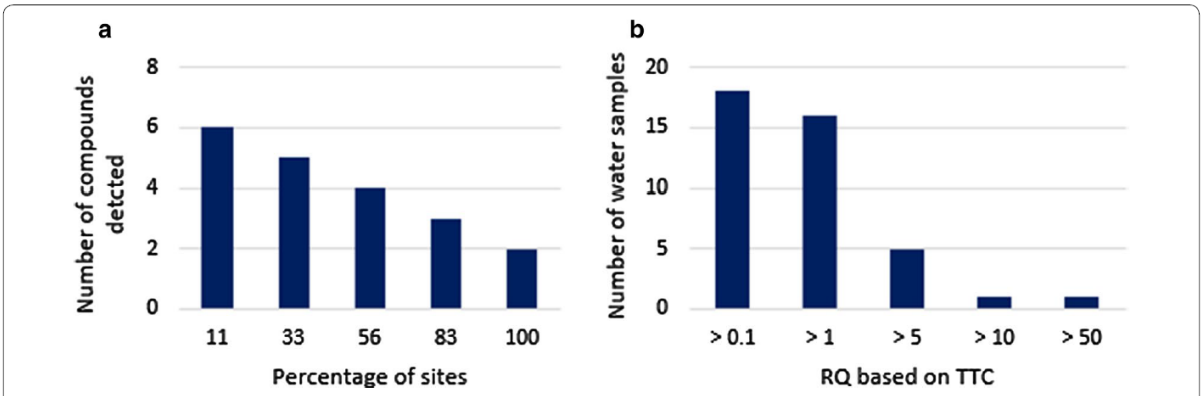

Fig. 4 **a** Co-occurrence of detected PSMs in between sites (18 positive samples) and **b** the number of samples exceeding mixture risk quotient (RQ) levels of PSMs

0.1 µg/L, mixture risks exceeded a RQ of 1 at 16 out of 18 sites, at 5 sites mixture RQ was above 5. At one site each, even RQs of 10 and 50 were exceeded (Fig. 4b). This may indicate that toxic risks by frequently occurring PSMs may not be negligible and should be included in risk assessment of chemical mixtures in water resources.

Conclusion

By target screening of 150 prioritized PSMs in river water from two small catchments in Germany, we were able to detect 12 compounds of different classes (e.g., coumarins, flavonoids, alkaloids and others), some of them occurring frequently. In many cases, concentrations of these compounds, which are known to exhibit substantial biological activity and possibly toxic effect, exceeded the concentrations of many anthropogenic chemicals in surface waters and TTC for drinking water individually and as mixture in almost all samples, in few cases by more than one order of magnitude. This finding clearly indicates that PSMs and other natural compounds should be included into monitoring and risk assessment and should be considered in the context of drinking water abstraction. A potential risk particularly during rain events promoting the leaching of PSMs to surface waters and massive occurrence of toxic plants in specific seasons may not be excluded. Large-scale seasonal target and suspect screening of PSMs together with toxicity testing of frequently occurring and high-concentration compounds is required to estimate the contribution of PSMs to overall water pollution and to identify seasons and situations potentially posing a risk to drinking water production. Toxic risks to aquatic ecosystems might be relevant, particularly in areas where vegetation undergoes drastic changes, for example by massive occurrence of toxic invasive species or by substantial changes in land use. Thus, we recommend to consider PSMs in monitoring and risk assessment of water resources. This is currently hampered by a lack of effect data including toxicity to aquatic organisms, endocrine disruption and genotoxicity and demands for biotesting of these compounds.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12302-020-00399-2.

Additional file 1. Samples, target compounds and internal standards.

Additional file 2. Data evaluation and results.

Abbreviations

LC–HRMS: Liquid chromatography coupled to high-resolution mass spectrometry; PSMs: Plant secondary metabolites; ELP: Elster, Luppe and Pleiße catchment; BD: Bode catchment; TTC: Threshold for toxicological concern; MS: Mass spectrometry; RQ: Risk quotient; MDLs: Method detection limit.

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Authors' contributions

MYN: conceptualization, investigation, experimental analysis, target compound selection, data evaluation and visualization, writing (original draft). MK: conceptualization, investigation, writing—review and editing; WB: conceptualization, supervision, writing—review and editing. CDS, BFG and TDB: in silico prediction and selection of target compounds, review and editing. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets obtained and analyzed in the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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Publisher's Note

Supplementary Material for

Target screening of plant secondary metabolites in river waters by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS)

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Table S1: Information on river samples and sampling locations.

SN	Sample Code	Catchment	Site	Sampling weather condition	Sampling Date	River name	Surrounding vegetation
1	ELP01	Leipzig	Floodplain forest	Rain event	01.05.2018	Tributary to Elsterflutbett	Forest plants
2	ELP02	Leipzig	Floodplain forest	Rain event	01.05.2018	Pleiße	Forest plants
3	ELP03	Leipzig	Floodplain forest	Rain event	01.05.2018	Elsterflutbett	Forest plants
4	ELP04	Leipzig	Floodplain forest	Rain event	01.05.2018	Paußnitz	Forest plants
5	ELP05	Leipzig	Floodplain forest	Rain event	01.05.2018	Elsterhochflutbelt	Forest plants
6	ELP06	Leipzig	Floodplain forest	Rain event	01.05.2018	Paußnitz	Forest plants
7	ELP07	Leipzig	Floodplain forest	Rain event	01.05.2018	Floßgraben(Batschke)	Forest plants
8	ELP08	Leipzig	Floodplain forest	Rain event	31.05.2018	Tributary to Elsterflutbett	Forest plants
9	ELP09	Leipzig	Floodplain forest	Rain event	31.05.2018	Paußnitz	Forest plants
10	ELP10	Leipzig	Floodplain forest	Rain event	31.05.2018	Elsterhochflutbelt	Forest plants
11	ELP11	Leipzig	Floodplain forest	Rain event	31.05.2018	Paußnitz	Forest plants
12	ELP12	Leipzig	Floodplain forest	Rain event	31.05.2018	Floßgraben(Batschke)	Forest plants
13	ELP13	Leipzig	Floodplain forest	Rain event	16.11.2018	Tributary to Elsterflutbett	Forest plants
14	ELP14	Leipzig	Floodplain forest	Rain event	16.11.2018	Tributary to Elsterflutbett	Forest plants
15	ELP15	Leipzig	Floodplain forest	Rain event	16.11.2018	Paußnitz	Forest plants
16	ELP16	Leipzig	Floodplain forest	Rain event	11.05.2019	Tributary to Elsterflutbett	Forest plants
17	ELP17	Leipzig	Floodplain forest	Rain event	18.03.2019	Tributary to Elsterflutbett	Forest plants
18	ELP18	Leipzig	Floodplain forest	Rain event	15.04.2019	Tributary to Elsterflutbett	Forest plants
19	ELP19	Leipzig	Floodplain forest	Rain event	11.05.2019	Tributary to Elsterflutbett	Forest plants
20	ELP20	Leipzig	Floodplain forest	Rain event	11.05.2019	Paußnitz	Forest plants
21	ELP21	Leipzig	Floodplain forest	Rain event	19.06.2019	Tributary to Elsterflutbett	Forest plants
22	ELP22	Leipzig	Floodplain forest	Rain event	19.06.2019	Paußnitz	Forest plants
23	ELP23	Leipzig	Southeast of Leipzig	Rain event	27.04.2018	Östliche Rietzchke	Agriculture + Natural
24	BD1	Bode	Minsleba	Rain event	02.07.2019	Holtemme	Agriculture
25	BD2	Bode	Minsleba	Rain event	02.07.2019	Barrenbach	Agriculture
26	BD3	Bode	Langeln	Rain event	02.07.2019	Deitzebach	Agriculture
27	BD4	Bode	Langeln	Rain event	02.07.2019	Osterbach	Agriculture
28	BD5	Bode	Steinerne Renne up stream	Rain event	02.07.2019	Holtemme	Forest plants
29	BD6	Bode	Schierke (upstream)	Rain event	02.07.2019	Kalt Bode	Forest plants
30	BD7	Bode	National park Harz	Rain event	02.07.2019	Wormke	Forest plants
31	BD8	Bode	Haberstadt	Rain event	02.07.2019	Holtemme	Agricuture + (WWTP)
32	BD9	Bode	Benzingerode	Rain event	02.07.2019	Hellbach	Forest + agriculture
33	BD10	Bode	B/n Hoym and Reinstedt	Rain event	02.07.2019	Getel	Agriclture
34	BD11	Bode	Wegeleben	Rain event	02.07.2019	Bode	Forest plants

35	BD12	Bode	Hasserode	Rain event	02.07.2019	Braunes Wasser	Forest plants
36	BD13	Bode	Drei Annen Hohne	Rain event	02.07.2019	Drängetalwasser	Forest plants
37	BD14	Bode	Elend	Rain event	02.07.2019	Spiebach	Forest plants
38	BD15	Bode	Sorge	Rain event	02.07.2019	Warme Bode	Forest plants
39	ELPD1	Leipzig	Floodplain forest	Dry (No rain event)	01.07.2018	Tributary to Elsterflutbett	Forest plants
40	ELPD2	Leipzig	Floodplain forest	Dry (No rain event)	01.07.2018	Paußnitz	Forest plants
41	ELPD3	Leipzig	Floodplain forest	Dry (No rain event)	01.07.2018	Paußnitz	Forest plants
42	ELPD4	Leipzig	Floodplain forest	Dry (No rain event)	01.07.2018	Floßgraben(Batschke)	Forest plants
43	ELPD5	Leipzig	Floodplain forest	Dry (No rain event)	20.07.2018	Elsterflutbett	Forest plants
44	ELPD6	Leipzig	Floodplain forest	Dry (No rain event)	20.07.2018	Elsterflutbett	Forest plants
45	ELPD7	Leipzig	Southeast of Leipzig	Dry (No rain event)	12.07.2018	Östliche Rietzchke	Agriculture + Natural vegetation
46	ELPD8	Leipzig	Southeast of Leipzig	Dry (No rain event)	12.08.2018	Östliche Rietzchke	Agriculture + Natural vegetation
47	BDD1	Bode	Minsleba	Dry (No rain event)	21.02.2018	Holtemme	Agriculture
48	BDD2	Bode	Minsleba	Dry (No rain event)	21.02.2018	Barrenbach	Agriculture
49	BDD3	Bode	Langeln	Dry (No rain event)	21.02.2018	Deitzebach	Agriculture
50	BDD4	Bode	Langeln	Dry (No rain event)	21.02.2018	Osterbach	Agriculture
51	BDD5	Bode	Steinerne Renne up stream	Dry (No rain event)	21.02.2018	Holtemme	Forest plants
52	BDD6	Bode	Schierke (upstream)	Dry (No rain event)	21.02.2018	Kalt Bode	Forest plants
53	BDD7	Bode	National park Harz	Dry (No rain event)	09.04.2018	Wormke	Forest plants
54	BDD8	Bode	Haberstadt	Dry (No rain event)	09.04.2018	Holtemme	Agricuture + (WWTP)
55	BDD9	Bode	Benzingerode	Dry (No rain event)	09.04.2018	Hellbach	Forest + agriculture
56	BDD10	Bode	B/n Hoym and Reinstedt	Dry (No rain event)	09.04.2018	Getel	Agriclture

Table S6: Plant metabolites selected for target screening of river samples.

SN	Compound	CAS-Number	Molecular formula	Supplier	Purity (%)	Example of plants producing the metabolite
1	7-Acetyllycopsamine	73544-48-6	C17H27NO6	Phytolab	93.23	Senecio L.
2	Aconitin	302-27-2	C34H47NO11	Phytolab	97.7	Aconitum
3	Ailanthone	981-15-7	C20H24O7	Sigma-Aldrich	> 98	Ailanthus
4	Ajmalicine	483-04-5	C21H24N2O3	Phytolab	99.81	Catharanthus
5	Anisatin	5230-87-5	C15H20O8	Phytolab	97.42	Illicium
6	Artemisinin	63968-64-9	C15H22O5	Phytolab	99.82	Artemisia
7	Atropine	55-48-1	C17H23NO3	Phytolab	99.2	Atropa, Brugmansia, Datura, Lycium, Mandragora, Scopolia
8	Baccatin III	27548-93-2	C31H38O11	Phytolab	98.18	Taxus
9	Berberine	2086-83-1	C20H18NO4+	Phytolab	98.67	Argemone, Berberis, Chelidonium, Corydalis, Mahonia, Papaver
10	Brucine	357-57-3	C23H26N2O4	Phytolab	99.39	Strychnos
11	Camptothecin	7689-03-4	C20H16N2O4	Phytolab	99.18	Camptotheca
12	Cevadine	62-59-9	C32H49NO9	Phytolab	98.21	Veratrum
13	(+)-Chelidonine	476-32-4	C20H19NO5	Phytolab	98.55	Chelidonium, Symphoricarpos
14	Colchicine	64-86-8	C22H25NO6	Phytolab	98.94	Colchicum
15	Convallatoxin	508-75-8	C29H42O10	Phytolab	86.45	Convallaria, Ornithogalum
16	(+)-Costunolide	553-21-9	C15H20O2	Phytolab	99.62	Inula, Laurus, Tanacetum
17	Cucurbitacin E	18444-66-1	C32H44O8	Phytolab	99.72	Anagallis, Bryonia, Cucurbita, Ecballium, Echinocystic, Gratiola, Iberis
18	Cytisin	485-35-8	C11H14N2O	Phytolab	100	Chamaecytisus, Genista, Laburnum, Sophora, Spartium, Ulex
19	10-Deacetylbaccatin III	32981-86-5	C29H36O10	Phytolab	99.29	Taxus
20	Daidzein	486-66-8	C15H10O4	Phytolab	99.28	Glycine, Pueraria, Trifolium
21	Diosgenin	512-04-9	C27H42O3	Phytolab	100	Asparagus, Maianthemum, Paris, Polygonatum, Solanum
22	Echimidine	520-68-3	C20H31NO7	Phytolab	93.13	Senecio L.
23	Echimidine N-oxide	41093-89-4	C20H31NO8	Phytolab	98.79	Senecio L.
24	Erucifoline	40158-95-0	C18H23NO6	Phytolab	99.87	Senecio L.
25	Erucifoline N-oxide	123864-94-8	C18H23NO7	Phytolab	99.68	Senecio L.

26	Europine	570-19-4	C16H27NO6	Phytolab	100	Senecio L.
27	Europine N-oxide	65582-53-8	C16H27NO7	Phytolab	99.08	Senecio L.
28	Formononetin	485-72-3	C16H12O4	Phytolab	99.24	Trifolium
29	Gelsemine	509-15-9	C20H22N2O2	Phytolab	99.23	Gelsemium
30	Gramine	87-52-5	C11H14N2	Sigma-Aldrich	99	Lupinus L.
31	Hederagenin	465-99-6	C30H48O4	Phytolab	96.75	Hedera
32	Heliotrine	303-33-3	C16H27NO5	Phytolab	91.23	Senecio L.
33	Heliotrine N-oxide	6209-65-0	C16H27NO6	Phytolab	100	Senecio L.
34	Homoharringtonine	26833-87-4	C29H39NO9	Phytolab	98.1	Cephalotaxus
35	Hordenine	539-15-1	C10H15NO	Phytolab	99.9	Hordeum
36	Huperzin A	102518-79-6	C15H18N2O	Phytolab	100	Huperzia
37	Hyoscyamine	620-61-1	C17H23NO3	Phytolab	100	Atropa, Brugmansia, Datura, Hyoscyamus, Mandragora, Scopolia
38	Imperatorin	482-44-0	C16H14O4	Phytolab	99.29	Ammi, Angelica, Heracleum, Levisticum, Pastinaca, Petroselinum, Peucedanum
39	Intermedine	10285-06-0	C15H25NO5	Phytolab	99.49	Senecio L.
40	Intermedine N-oxide	95462-14-9	C15H25NO6	Phytolab	100	Senecio L.
41	Isopimaric Acid	5835-26-7	C20H30O2	Sigma-Aldrich	>98	Pinus
42	(+)-Isocorydin	475-67-2	C20H23NO4	Phytolab	100	Argemone, Berberis, Corydalis, Glaucium, Isopyrum, Mahonia, Papaver
43	Isopimpinellin	482-27-9	C13H10O5	Phytolab	99.18	Ammi, Angelica, Apium, Heracleum, Pastinaca, Petroselinum, Pimpinella, Ruta
44	Jacobine	6870-67-3	C18H25NO6	Phytolab	100	Senecio L.
45	Jacobine N-oxide	38710-25-7	C18H25NO7	Phytolab	98.43	Senecio L.
46	Jervine	469-59-0	C27H39NO3	Phytolab	99.19	Veratrum
47	Juglone	481-39-0	C10H6O3	Phytolab	99.61	Juglans
48	Lasiocarpine	303-34-4	C21H33NO7	Phytolab	100	Senecio L.
49	Lasiocarpine N-oxide	127-30-0	C21H33NO8	Phytolab	97.39	Senecio L.
50	Lathyrol	34420-19-4	C20H30O4	Phytolab	99.62	Euphorbia
51	Lycopsamine	10285-07-1	C15H25NO5	Phytolab	99.12	Senecio L.
52	Lycopsamine N-oxide	95462-15-0	C15H25NO6	Phytolab	92.59	Senecio L.
53	Lycorine	476-28-8	C16H17NO4	Sigma-Aldrich	>98	Galanthus, Leucojum, Narcissus

54	Matrine	519-02-8	C15H24N2O	Phytolab	99.62	Sophora
55	Monocrotaline	315-22-0	C16H23NO6	Phytolab	100	Senecio L.
56	Narciclasine	29477-83-6	C14H13NO7	Phytolab	99.58	Narcissus
57	Nicotine	65-31-6	C10H14N2	Phytolab	100	Asclepias, Equisetum, Huperzia, Lycopodium, Nicotiana, Sedum
58	(-)-Nuciferine	475-83-2	C19H21NO2	Phytolab	99.72	Nymphaea
59	Oleandrin	465-16-7	C32H48O9	Phytolab	97.95	Nerium, Ornithogalum
60	Parthenolid	20554-84-1	C15H20O3	Phytolab	94.62	Tanacetum, Ambrosia
61	Papaverin	58-74-2	C20H21NO4	Phytolab	100	Papaver
62	Picrotoxinin	17617-45-7	C15H16O6	Phytolab	99.1	Anamirta
63	Pilocarpine	54-71-7	C11H16N2O2	Phytolab	100	Pilocarpus
64	Primin	15121-94-5	C12H16O3	Phytolab	98.77	Primula
65	8-Prenylnaringenin	53846-50-7	C20H20O5	Phytolab	100	Humulus
66	Protopin	130-86-9	C20H19NO5	Phytolab	100	Argemone, Chelidonium, Corydalis, Eschscholzia, Fumaria, Glaucium, Lamprocapnos, Papaver
67	Pterosin B	34175-96-7	C14H18O2	Phytolab	99.8	Pteridium
68	(-)-Reserpine	50-55-5	C33H40N2O9	Phytolab	99.9	Vinca
69	Retrorsine	480-54-6	C18H25NO6	Phytolab	98.95	Senecio L.
70	Retrorsine N-oxide	15503-86-3	C18H25NO7	Phytolab	96.59	Senecio L.
71	Sanguinarine	5578-73-4	C20H14NO4+	Phytolab	98.59	Argemone, Chelidonium, Eschscholzia, Fumaria, Glaucium, Lamprocapnos, Papaver
72	alpha-Santonin	481-06-1	C15H18O3	Phytolab	98.7	Artemisia
73	(-)-Scopolamine	114-49-8	C17H21NO4	Phytolab	98.66	Atropa, Brugmansia, Datura, Hyoscyamus, Mandragora, Scopolia
74	Senkirkine	2318-18-5	C19H27NO6	Phytolab	98.39	Senecio L.
75	Senecionine	130-01-8	C18H25NO5	Phytolab	99.42	Senecio L.
76	Senecionine N-oxide	13268-67-2	C18H25NO6	Phytolab	99.71	Senecio L.
77	Seneciphylline	480-81-9	C18H23NO5	Phytolab	99.73	Senecio L.
78	Seneciphylline N-oxide	38710-26-8	C18H23NO6	Phytolab	100	Senecio L.
79	Senecivernine	72755-25-0	C18H25NO5	Phytolab	98.22	Senecio L.
80	Senecivernine N-oxide	101687-28-9	C18H25NO6	Phytolab	99.84	Senecio L.
81	Solasodin	126-17-0	C27H43NO2	Phytolab	100	Solanum

82	Sophocarpine	6483-15-4	C15H22N2O	Phytolab	99.86	Sophora
83	(-)-Sparteine	90-39-1	C15H26N2	Sigma-Aldrich	>98	Chamaecytisus, Cytisus, Genista, Laburnum, Lupinus, Spartium
84	Strophanthidin	66-28-4	C23H32O6	Phytolab	95.84	Adonis, Convallaria, Coronilla, Erysimum, Ornithogalum, Sisymbrium
85	(-)-Strychnine	57-24-9	C21H22N2O2	Phytolab	98.07	Strychnos
86	Taxol	33069-62-4	C47H51NO14	Phytolab	98.54	Taxus
87	Tetrahydropalmatin	3520-14-7	C21H25NO4	Phytolab	99.78	Corydalis
88	Umbelliferone	93-35-6	C9H6O3	Phytolab	99.33	Ruta
89	Vincamin	1617-90-9	C21H26N2O3	Sigma-Aldrich	98	Vinca
90	Yohimbine	65-19-0	C21H26N2O3	Phytolab	99.62	Pausinystalia
91	(+)-Lupanine	550-90-3	C15H24N2O	Biozol/TRC	97	Lupinus spp.
92	(+)-Sparteine	492-08-0	C15H26N2	SigmaAldrich	>80	Lupinus mutabilis
93	[6]-Gingerol	23513-14-6	C17H26O4	Geyer/J&K	98	Zingiber officinale
94	2-Hydroxycinnamic acid	614-60-8	C9H8O3	Aldrich	97	Citrus spp.
95	2-O-Methyladenosine	2140-79-6	C11H15N5O4	Geyer/Alfa Aesar	99	mycelia of Cordyceps sinensis
96	4-Hydroxycoumarin	1076-38-6	C9H6O3	Ehrenstorfer	99.5	Ruta
97	Aescin	8047-15-2	C55H86O24	Sigma/Geyer	≥95	Aesculus hippocastanum
98	Akebia saponin D	39524-08-6	C47H76O18	Sigma	98	Dipsacus asper
99	Aloin A	1415-73-2	C21H22O9	Sigma-Aldrich	≥ 97	Aloe barbadensis
100	alpha-Cyperone	473-08-5	C15H22O	Biozol/Medchemexpress	>98	Cyperus rotundus (coco-grass, Java grass, nut grass, purple nut sedge or purple nutsedge, red nut sedge, Khmer kravanh chruk)
101	Alpinetin	1090-65-9	C16H14O4	Phytolab	95	Alpinia spp
102	Anabasine	13078-04-1	C10H14N2	Aldrich	97	Anabasis aphylla
103	Apiin	26544-34-3	C26H28O14	Phytolab	≥97.0	parsley and celery
104	Aspirin	50-78-2	C9H8O4	Geyer/HPC	99.9	white willow (Salix alba)
105	Bergapten/Heraclin	484-20-8	C12H8O4	Geyer/Sigma	99	genus Heracleum in the family Apiaceae - Heracleum grandiflorum
106	Chrysin	480-40-0	C15H10O4	Sigma-Aldrich	≥ 98	Artemisia campestris
107	Citrinin	518-75-2	C13H14O5	Geyer/J&K	98	Penicillium citrinum
108	Conessine	546-06-5	C24H40N2	Geyer/Sigma	97	Holarrhena floribunda, Holarrhena antidysenterica

109	Coniferaldehyde	458-36-6	C10H10O3	Geyer/Sigma	98	Quercus spp.
110	Coniine	3238-60-6	C8H17N	Carbolution	95	Conium maculatum
111	Coumarin	91-64-5	C9H6O2	Merck	≥ 99	tonka bean (Dipteryx odorata), Picea abies
112	Crotonoside	1818-71-9	C10H13N5O5	Geyer/J&K	98	Croton tiglium
113	Daphnetin	486-35-1	C9H6O4	Geyer/J&K	98	Daphne oleoides
114	DIBOA	17359-54-5	C8H7NO4	Biozol/TRC	98	Zea mays
115	Digitoxigenin	143-62-4	C23H34O4	Carbolution	97	Digitalis purpurea
116	Digoxin	20830-75-5	C41H64O14	SigmaAldrich	95	Digitalis orientalis
117	DIMBOA	15893-52-4	C9H9NO5	Biozol/TRC	97	Zea mays L.
118	Dimethylfraxetin	6035-49-0	C12H12O5	Biozol/Medchemexpress	>98	Fraxinus excelsior
119	Embelin	550-24-3	C17H26O4	Carbolution/AkSci	98	Embelia ribes
120	Emodin	481-72-1	C15H10O5	Biozol/ChemScene	98	Reynoutria japonica, Japanese knotweed (Reynoutria japonica syn. Polygonum cuspidatum)
121	Epigallocatechin	970-74-1	C15H14O7	Biozol/TRC	97	Vicia faba
122	Esculetin	305-01-1	C9H6O4	Geyer/J&K	98	Fraxinus excelsior
123	Fraxetin	574-84-5	C10H8O5	Biozol/Targetmol	>98	Fraxinus spp.
124	Fraxidin	525-21-3	C11H10O5	Geyer/Roth	95	Fraxinus excelsior
125	Galantamine	357-70-0	C17H21NO3	Sigma	94	Galanthus caucasicus (Caucasian snowdrop), Galanthus woronowii (Voronov's snowdrop)
126	Gibberellin A4	468-44-0	C19H24O5	Geyer/Sigma	90	Alstroemeria hybrida, Brassica napus
127	Glucobrassicin	4356-52-9	C16H20N2O9S2	Phytolab	90	Brassica napus
128	Goitrin	13190-34-6	C5H7NOS	Biozol/Targetmol	>98	Ruciferous vegetables such as cabbage, brussels sprouts and rapeseed oil
129	Gramine	87-52-5	C11H14N2	SigmaAldrich	99	Lupinus spp.
130	Guanosine	118-00-3	C10H13N5O5	Sigma	≥98	Zea mays L.
131	Indole-3-acetic acid	87-51-4	C10H9NO2	Aldrich	98	Picea abies, Zea mays, Brassica oleracea, Quercus robur
132	Indole-3-acrylic acid	1204-06-4	C11H9NO2	Chempur	98	Lens culinaris
133	Indole-3-carboxaldehyde	487-89-8	C9H7NO	Aldrich	97	*Brassica oleracea* (Cauliflower, broccoli, Brussels sprouts, cabbage, collard greens, and kale)
134	Kaempferitrin	482-38-2	C27H30O14	Phytolab	≥97	*Hedyotis verticillata* and *Onychium japonicum*
135	Matairesinol	580-72-3	C20H22O6	Geyer/Sigma	95	Plant lignans occurring in a variety of different foods, e.g., oilseeds,whole grains, vegetables, and fruits

136	Myrtenal	564-94-3	C10H14O	Aldrich	98	broad bean (*Vicia faba*)
137	Paprazine	36417-86-4	C17H17NO3	Phytolab	95	Vicia faba
138	Piperine	94-62-2	C17H19NO3	Sigma-Aldrich	97	*Piper nigrum*
139	Psoralen	66-97-7	C11H6O3	Geyer/J&K	99	Psoralea corylifolia L., Heracleum
140	Ptaquiloside	87625-62-5	C20H30O8	Lab extracted from Bracken Fern	-	Pteridium aquilinum, Bracken Fern
141	Pterosin A	35910-16-8	C15H20O3	Lab extracted from Bracken Fern	-	Pteridium aquilinum, Bracken Fern
142	Pterosin G	35964-50-2	C14H18O3	Lab extracted from Bracken Fern	-	Pteridium aquilinum, Bracken Fern
143	Ptesculentoside		C20H30O9	Lab extracted from Bracken Fern	-	Pteridium esculentum, Pteridium aquilinum, Bracken Fern
144	Quercetin	6151-25-3	C15H14O9	Roth	98	Fruits and vegetables (e.g. Brassica oleracea)
145	Rutin	250249-75-3	C27H30O16	Alfa Aesar	97	Ruta graveolens
146	Scopoletin	92-61-5	C10H8O4	Sigma-Aldrich	97	Genus Scopolia such as Scopolia carniolica and Scopolia japonica
147	Theobromine	83-67-0	C7H8N4O2	Geyer/Roth	≥ 99	Theobroma cacao
148	Tomatidine	77-59-8	C27H45NO2	Biozol/ChemScene	98	Tomato Plant
149	trans-Zeatin	1637-39-4	C10H13N5O	Carbolution/AkSci	98	Zea mays
150	Ursolic acid	77-52-1	C30H48O3	Carbolution/AkSci	98	Mirabilis jalapa
151	Valerophenone	1009-14-9	C11H14O	Geyer/J&K	99	**Celery** (*Apium graveolens*)

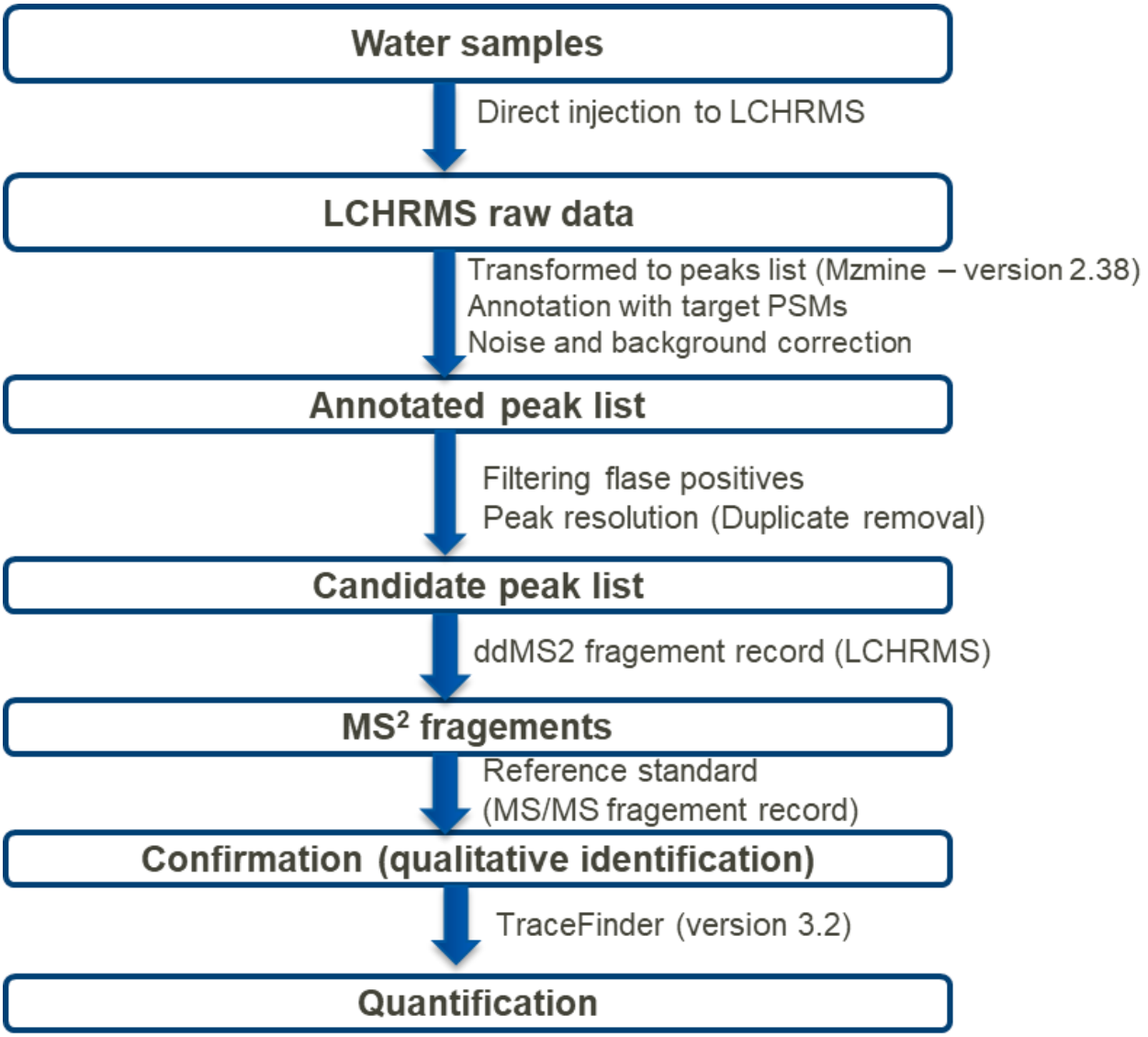

Figure S27: Workflow for screening toxic plant metabolites in river waters.

Table S7: Setting for MZmine data processing.

	Parameters	Mass detection	Chromatogram building	Chromatogram deconvolution	Join aligner	Gap filling	Target annotation (identification)
Peak detection	Mass detector	Centroid					
	Noise level	5.00E+3					
	MS level	1.0					
	Group intensity threshold		1.00E+4				
	Min height intensity		5.00E+03				
	m/z tolerance		0.001				
	Algorithm			Local minimum search			
	Chromatographic threshold (%)			60.0			
	Search minimum in retention time range			0.1			
	Minimum relative height (%)			30			
	Minimum absolute height			5.0E+4			
	Min ratio of peak top/edge			2.3			
	Peak duration range (min)			0.1-0.5			
Peak alignment and identification	m/z tolerance				0.001	0.001	0.001
	Weight for m/z tolerance				70		
	Retention time tolerance (absolute, min)				0.3	0.15	0.5
	Weight for RT				30		
	Intensity tolerance (%)					30.00	
	RT range						
	Adducts						M+H+, M+Na+, M+NH4+,

Table S4: Internal standards used for the chemical analysis (ESIpos)

			Used ions (ESI+)		
ID	**Compound name**	**Monoisotopic mass**	**M+**	**M+H+**	**M+NH4+**
IS03	IS03_Mono-isobutylphthalate-D4	226.1143		227.1216	
IS04	IS04_Creatinine-D3	116.0777		117.085	
IS05	IS05_Diazinon-D10	314.1638		315.1711	
IS06	IS06_Benzophenone-3-D5	233.11		234.1173	
IS07	IS07_p-Toluene-sulfonamide-D4	175.0605		176.0678	193.0933
IS10	IS10_1-Naphthol-D7	151.1015			
IS13	IS13_Cotinine-D3	179.1138		180.1211	
IS16	IS16_Bisphenol A D16	244.2155			
IS17	IS17_Diglyme-D6	140.132		141.1392	
IS18	IS18_4-Nitrophenol-D4	143.0521			
IS19	IS19_Chlormequat-D9	131.1296	131.13		
IS22	IS22_Carbamazepine-D10	246.1577		247.165	
IS23	IS23_Triclosan-D3	290.97			
IS24	IS24_Atrazine-13C3	218.1038		219.1111	
IS25	IS25_Estradiol-D3	275.1965			

IS27	IS27_4-Nonylphenol-D4	224.2078			
IS28	IS28_Benzotriazole-D4	123.0735		124.0807	
IS29	IS29_Carbendazim-D4	195.0946		196.1019	
IS30	IS30_Tri-n-butylphosphate-D27	293.3342		294.3414	
IS31	IS31_DEET-D7	198.175		199.1822	
IS37	IS37_Metolachlor-D6	289.1716		290.1788	
IS38	IS38_Isoproturon-D3	209.1607		210.168	
IS39	IS39_Mecoprop-D3	217.0585			
IS40	IS40_Diclofenac-D4	299.0418		300.0491	
IS41	IS41_Caffeine-D3	197.0992		198.1065	
IS42	IS42_Clarithromycin-D3	750.4957		751.503	
IS43	IS43_Desisopropylatrazine-D5	178.0782		179.0855	
IS44	IS44_Decyltrimethylammonium-D30	230.4256	230.43		
IS46	IS46_Laurylsulfate-D25	291.3121			
IS47	IS47_Atenolol-D7	273.207		274.2143	
IS48	IS48_Progesterone-D9	323.2811		324.2883	
IS49	IS49_Verapamil-D6	460.3208		461.3281	
IS50	IS50_Bezafibrate-D4	365.1332		366.1405	
IS51	IS51_Sulfamethoxazole-D4	257.0772		258.0845	
IS54	IS54_Acesulfame-D4	167.019			
IS55	IS55_Tebuconazole-D9	316.2016		317.2089	
IS56	IS56_Hydrochlorothiazide-13C6	302.9846			
IS57	IS57_Imidacloprid-D4	259.0774		260.0847	
IS62	IS62_Bentazone-D6	246.09452			
IS63	IS63_Cyclamate-D11	190.13066			

Table S8: Mass spectrometry setting for the chemical analysis of water samples.

Parameters	**Full MS**	**MS/MS**
Polarity	positive	positive
Resolving power (m/z 200)	70,000	35,000
Automated gain control target value	3e6	5e5
Maximum injection time	200 ms	110 ms
HCD collision energy		45 a.u

Table S9: Quantifier and confirming ions used in TraceFinder for quantification of detected target compounds.

Compound Name	Chemical formula	CAS	Quantifier MS1 (Precursor) m/z (M+H+)	Adduct	Polarity	Retention time (min)	Confirming ion – 1 (MS2, M+H+)	Confirming ion – 2 (MS2, M+H+)
Coumarin	$C_9H_6O_2$	91-64-5	147.0441	M+H	+	7.3	103.0542	91.0542
Esculetin	$C_9H_6O_4$	305-01-1	179.0336	M+H	+	4.2	133.0283	123.044
Fraxidin	$C_{11}H_{10}O_5$	525-21-3	223.06	M+H	+	7.8	190.026	162.0311
Scopoletin	$C_{10}H_8O_4$	92-61-5	193.0496	M+H	+	7.1	133.0283	165.0545
Bergapten	$C_{12}H_8O_4$	484-20-8	217.0495	M+H	+	10.1	202.0259	174.031
Psoralen	$C_{11}H_6O_3$	66-97-7	187.0388	M+H	+	9.1	143.049	131.049
Lycorine	$C_{16}H_{17}NO_4$	476-28-8	288.1225	M+H	+	1	177.0546	119.0491
Narciclasine	$C_{14}H_{13}NO_7$	29477-83-6	308.0765	M+H	+	5.7	248.0553	214.0499
Nicotine	$C_{10}H_{14}N_2$	54-11-5	163.1228	M+H	+	0.9	132.0807	106.0651
Piperine	$C_{17}H_{19}NO_3$	94-62-2	286.1434	M+H	+	11.9	201.0544	135.0439
Formononetin	$C_{16}H_{12}O_4$	485-72-3	269.0804	M+H	+	10.8	237.0544	213.0908
Coniferyl aldehyde	$C_{10}H_{10}O_3$	458-36-6	179.0701	M+H	+	7.6	147.044	119.0492

Table S10: The concentration of detected metabolites in river water from Leipzig and Bode catchment.

Sample ID	Sampling season	Sampling location	Concentration (ng/l)												Number of SPMs detected per sample	Grand total (all SPMs detected) concentration (ng/l) per sample
			Bergapten	Coniferyl aldehyde	Coumarin	Esculetin	Formononetin	Fraxidin	Lycorine	Narciclasine	Nicotine	Piperine	Psoralen	Scopoletin		
ELP19	Summer	Leipzig	ND	ND	ND	ND	8	546	ND	ND	ND	271	ND	47	4	872
ELP23	Spring	Leipzig	510	46	ND	ND	ND	56	ND	ND	ND	338	ND	31	5	981
ELP17	Spring	Leipzig	ND	13	ND	ND	ND	294	2331	506	ND	ND	ND	13	5	3157
ELP18	Summer	Leipzig	ND	ND	ND	1658	ND	1144	1015	3352	ND	ND	ND	41	5	7211
ELP2	Spring	Leipzig	ND	ND	ND	ND	12	84	ND	ND	ND	1	ND	18	4	115
ELP3	Spring	Leipzig	ND	ND	ND	ND	23	173	ND	ND	2	ND	ND	9	4	207
ELP9	Summer	Leipzig	ND	ND	ND	ND	33	132	ND	ND	ND	ND	ND	21	3	186
ELP10	Spring	Leipzig	ND	ND	12	ND	35	196	ND	ND	6	1	ND	34	6	284
ELP21	Spring	Leipzig	ND	ND	ND	116	ND	228	ND	ND	ND	ND	ND	20	3	363
BD2	Summer	Bode	ND	ND	ND	ND	ND	52	ND	ND	35	ND	ND	47	3	134
BD8	Summer	Bode	ND	ND	ND	ND	ND	130	ND	ND	ND	294	ND	13	3	436
BD9	Summer	Bode	ND	ND	ND	ND	ND	85	ND	ND	ND	ND	224	13	3	321
BD10	Summer	Bode	541	ND	43	104	ND	106	ND	ND	ND	ND	141	49	6	984
BD1	Summer	Bode	ND	ND	ND	ND	ND	36	ND	ND	ND	ND	ND	8	2	44
BD3	Summer	Bode	ND	ND	ND	113	123	59	11	ND	ND	ND	ND	38	5	343
BD4	Summer	Bode	ND	ND	ND	ND	ND	155	ND	ND	4	4	ND	27	4	190
BD5	Summer	Bode	ND	ND	ND	ND	ND	19	ND	ND	ND	ND	ND	7	2	25
BD12	Spring	Bode	ND	ND	ND	157	ND	ND	ND	ND	ND	ND	ND	23	2	179

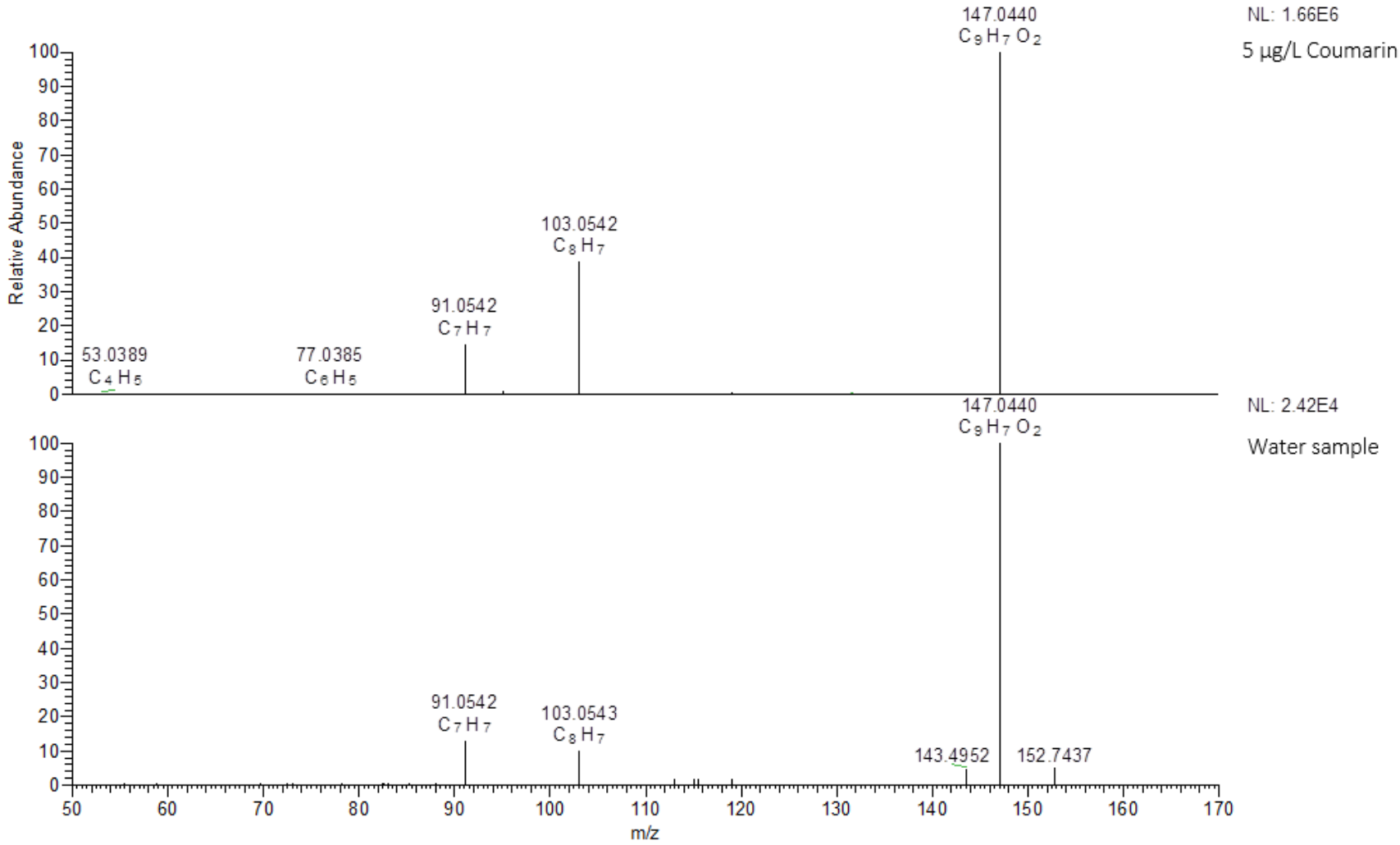

Figure S28: MS/MS spectra (HCD fragmentation at 45 a.u.) of coumarin in a reference standard and water sample (NL: signal intensity at 100% relative abundance).

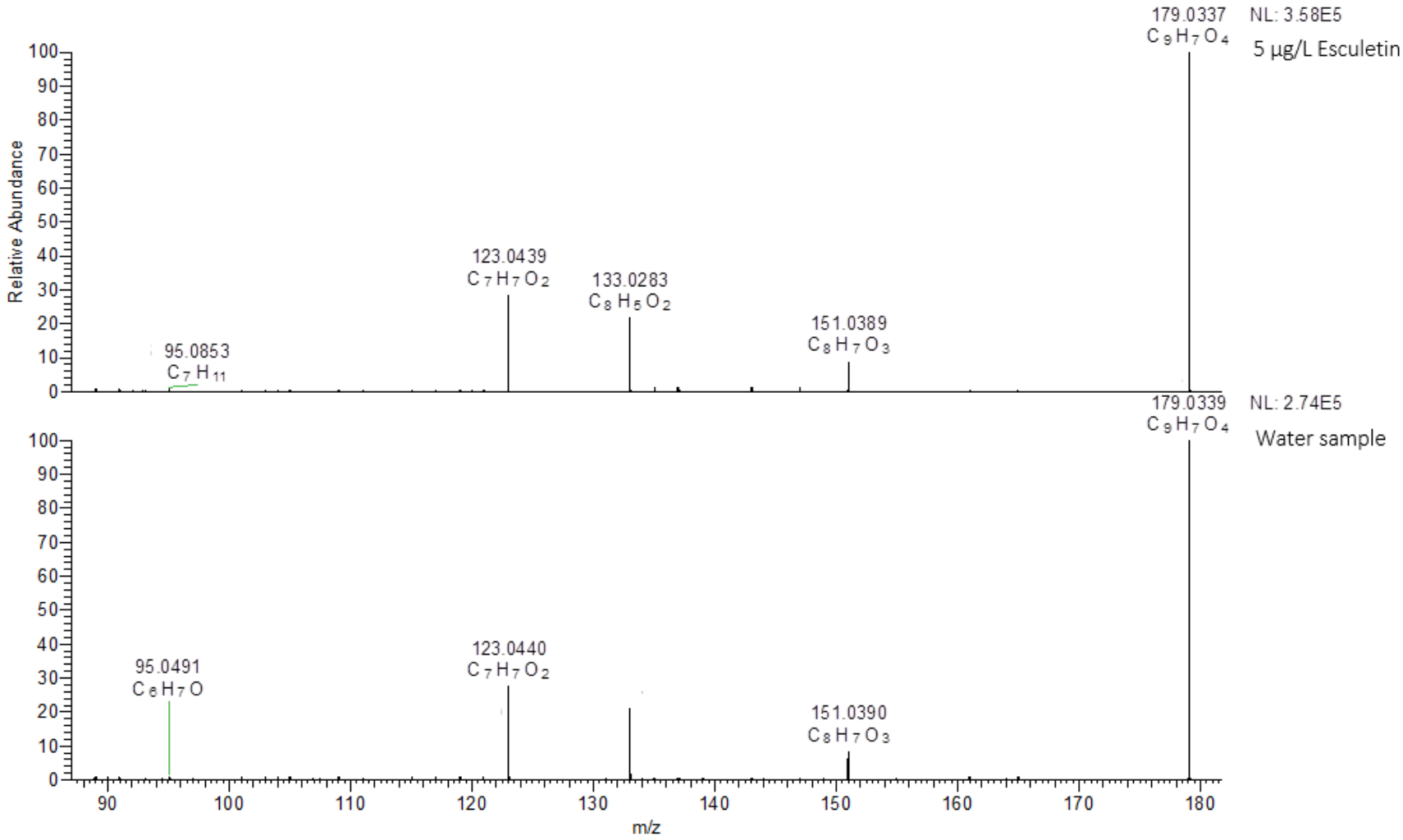

Figure S29: MS/MS spectra (HCD fragmentation at 45 a.u.) of esculetin in a reference standard and water sample (NL: signal intensity at 100% relative abundance).

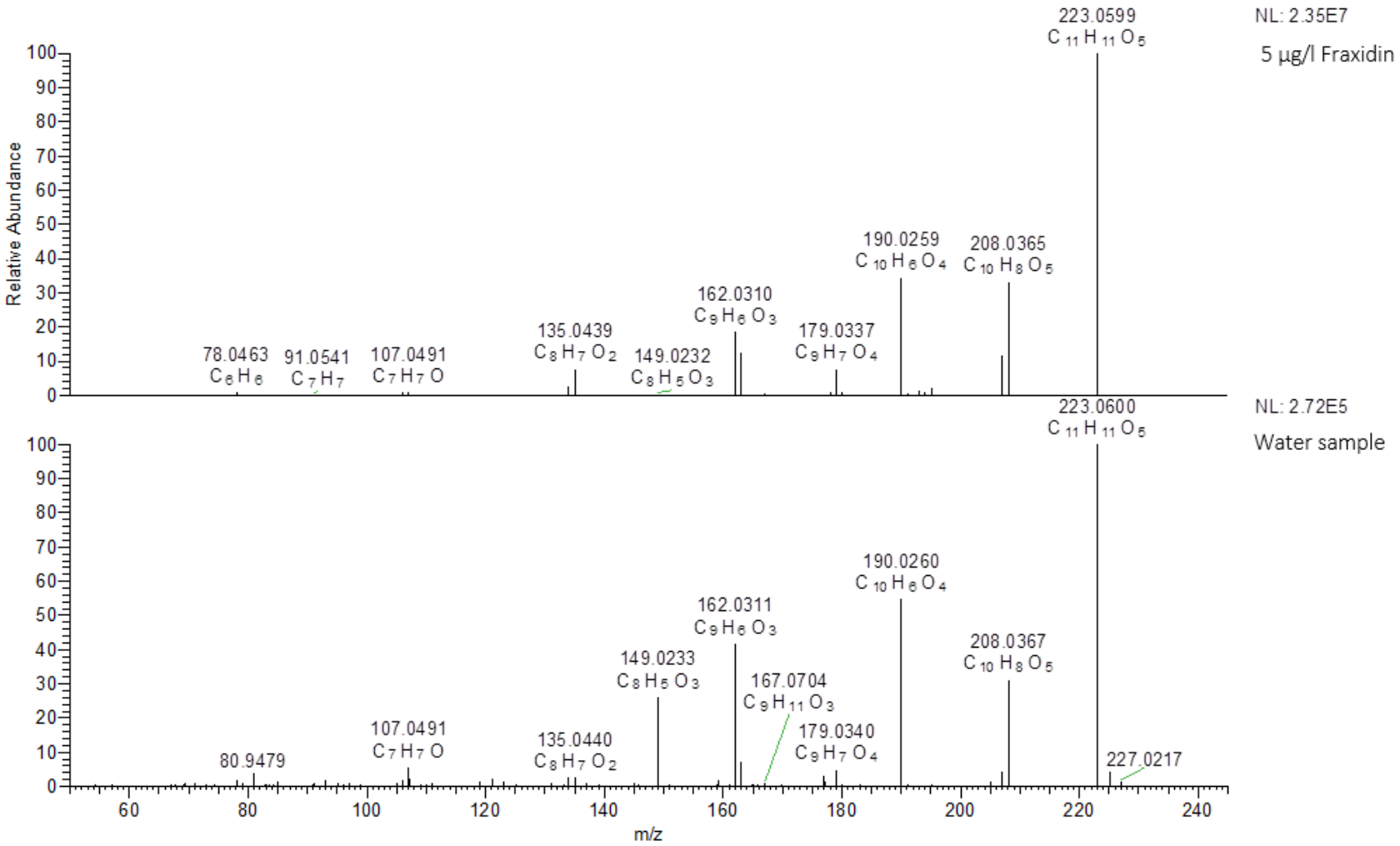

Figure S30: MS/MS spectra (HCD fragmentation at 45 a.u.) of fraxidin in a reference standard and water sample (NL: signal intensity at 100% relative abundance).

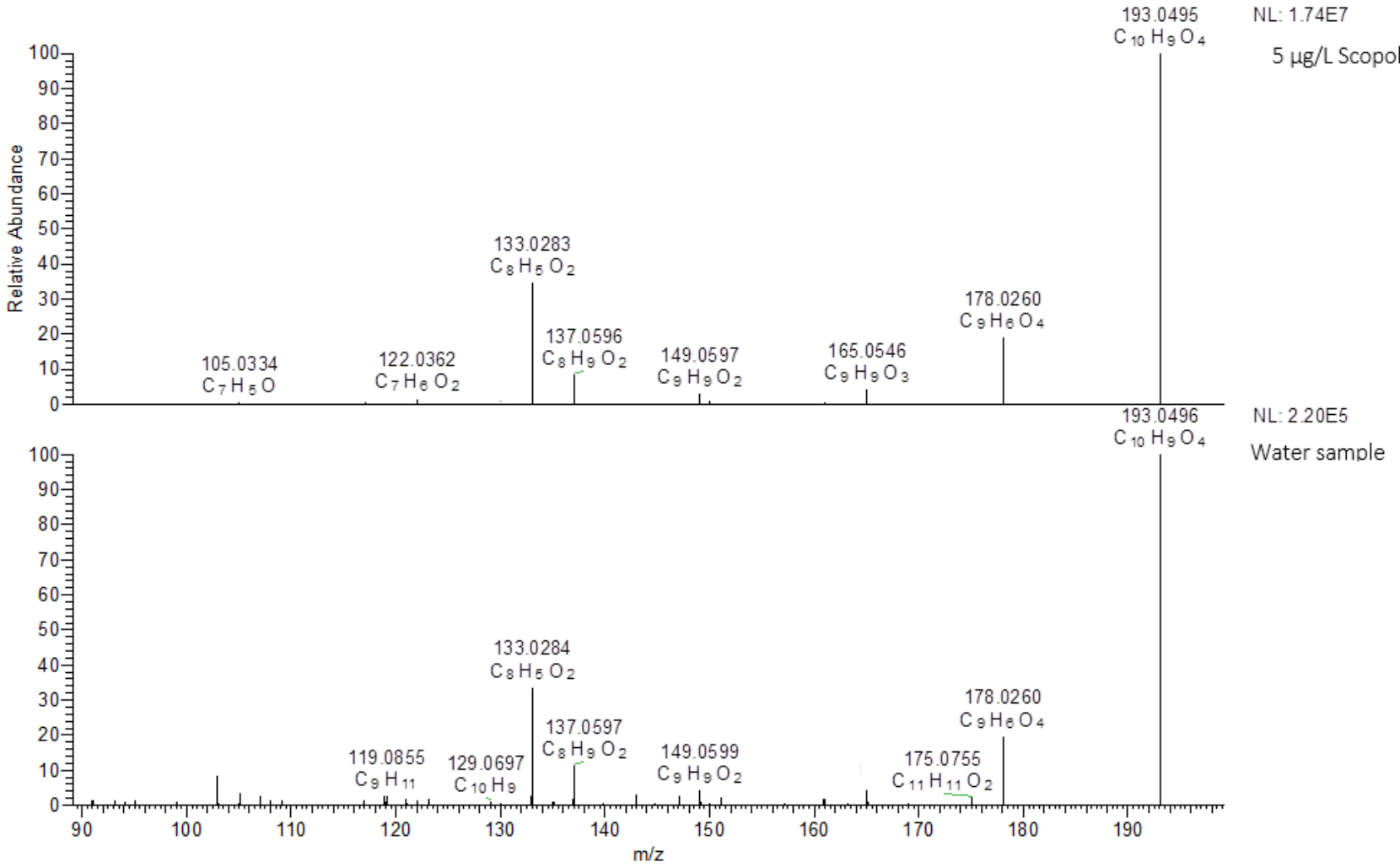

Figure S31: MS/MS spectra (HCD fragmentation at 45 a.u.) of scopoletin in a reference standard and water sample (NL: signal intensity at 100% relative abundance).

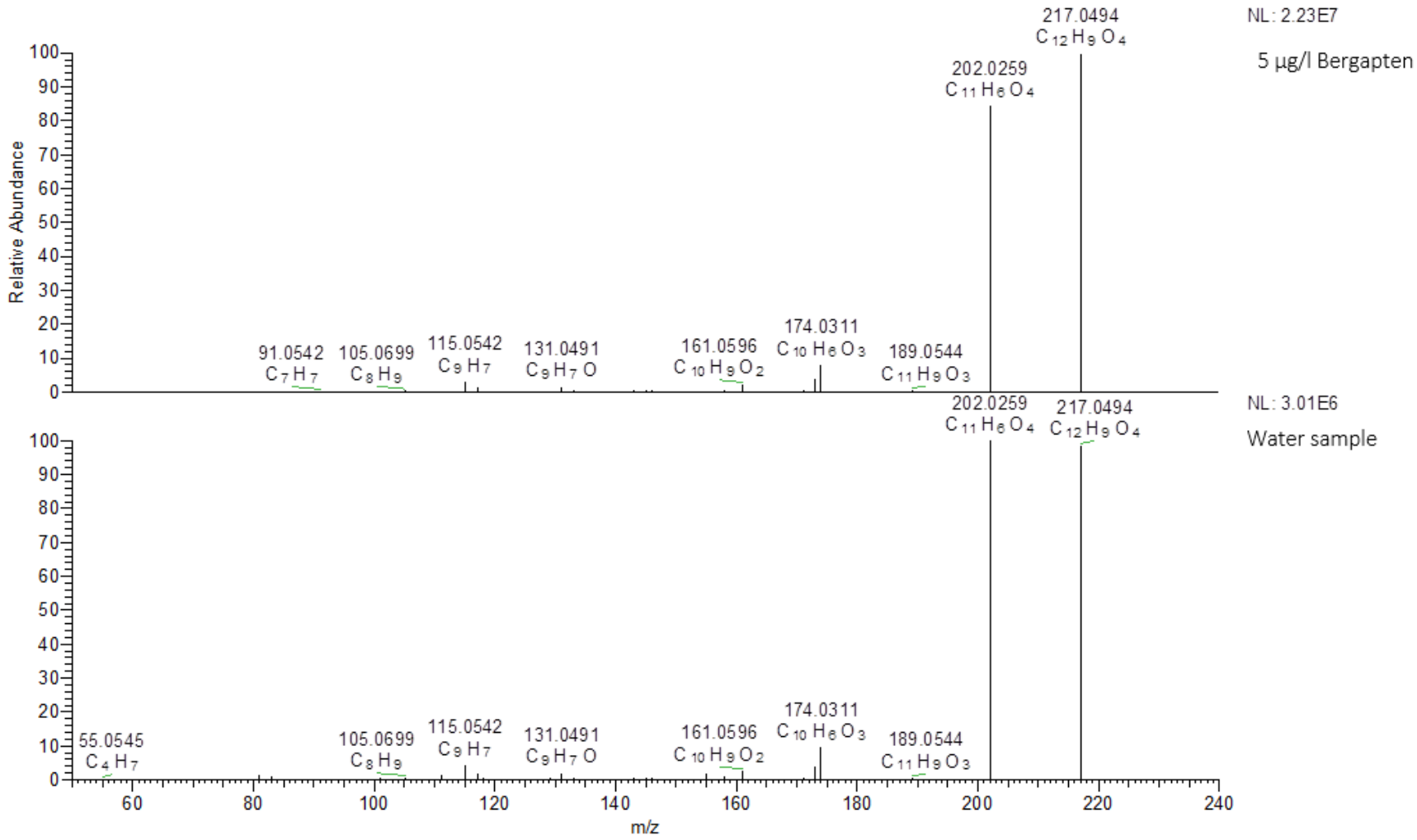

Figure S32: MS/MS spectra (HCD fragmentation at 45 a.u.) of bergapten in a reference standard and water sample (NL: signal intensity at 100% relative abundance).

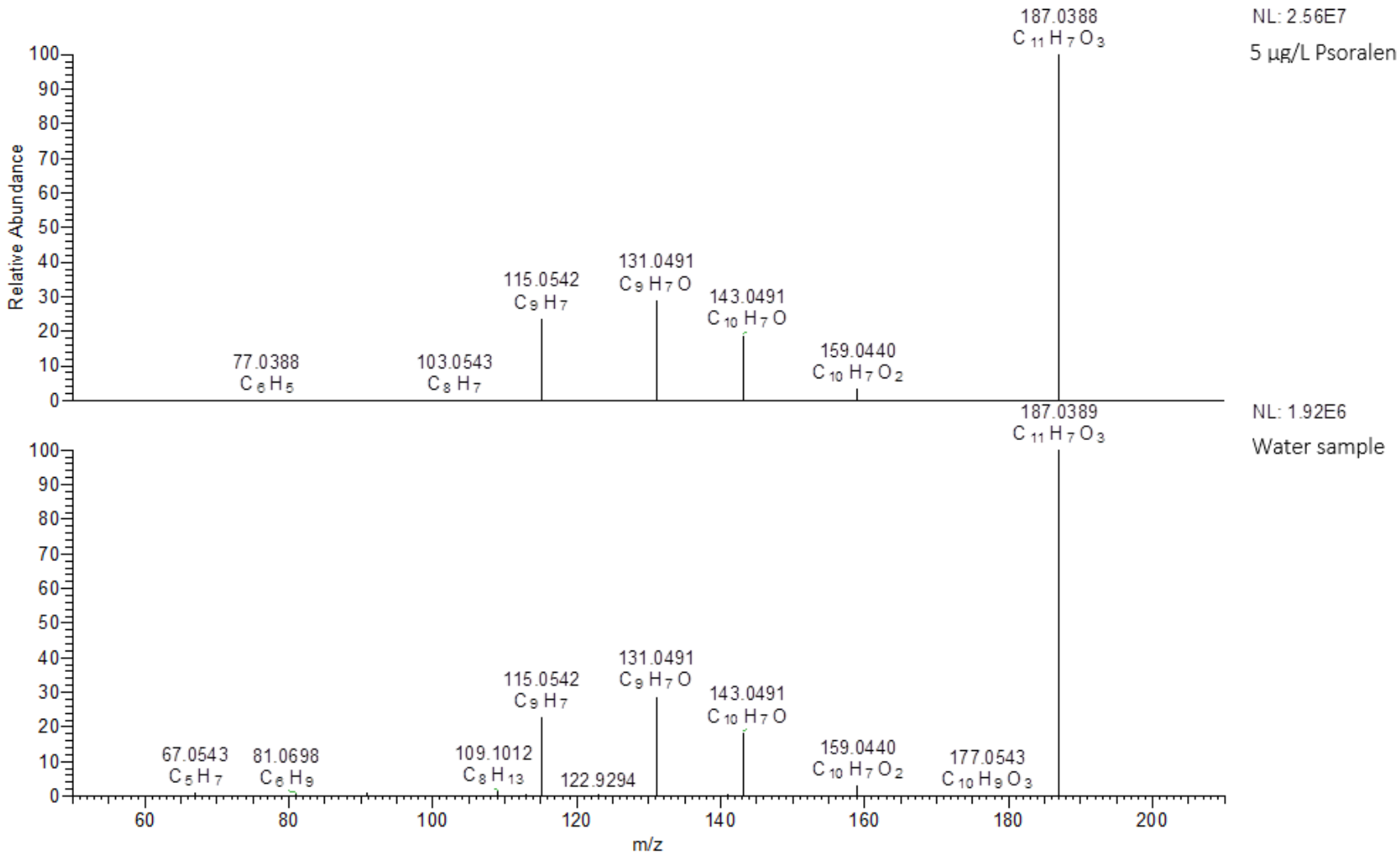

Figure S33: MS/MS spectra (HCD fragmentation at 45 a.u.) of psoralen in a reference standard and water sample (NL: signal intensity at 100% relative abundance).

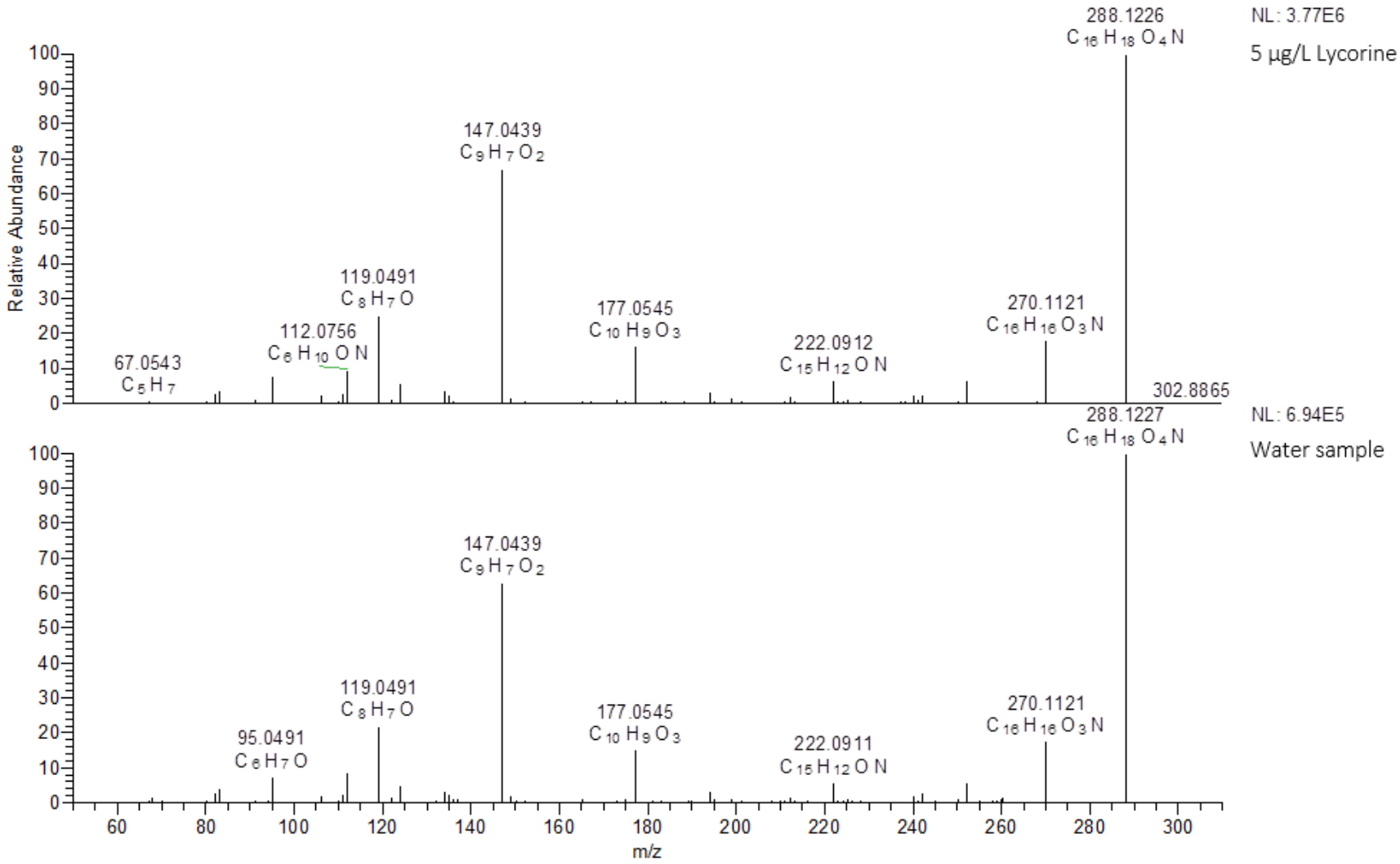

Figure S34: MS/MS spectra (HCD fragmentation at 45 a.u.) of lycorine in a reference standard and water sample (NL: signal intensity at 100% relative abundance).

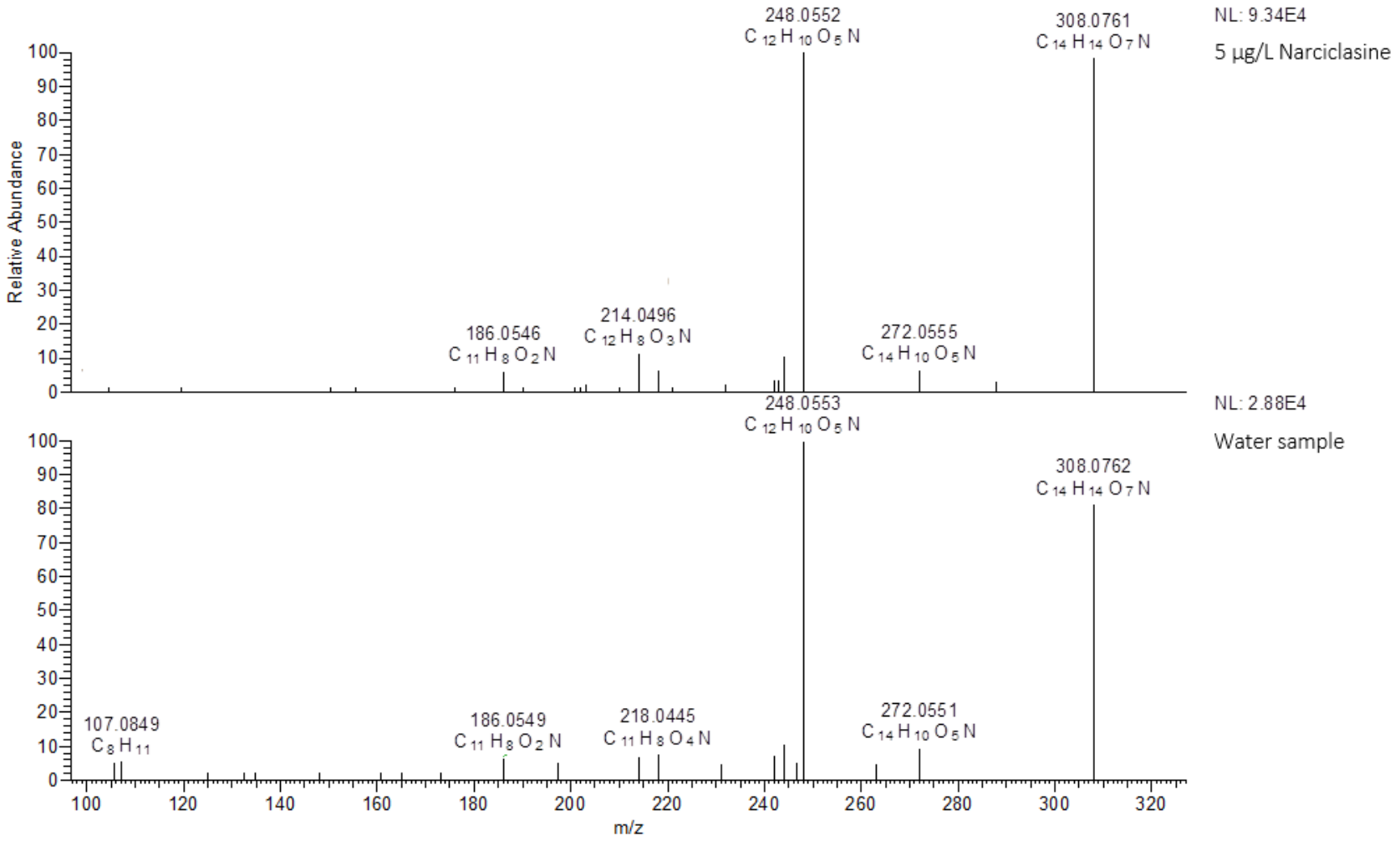

Figure S35: MS/MS spectra (HCD fragmentation at 45 a.u.) of narciclasine in a reference standard and water sample (NL: signal intensity at 100% relative abundance).

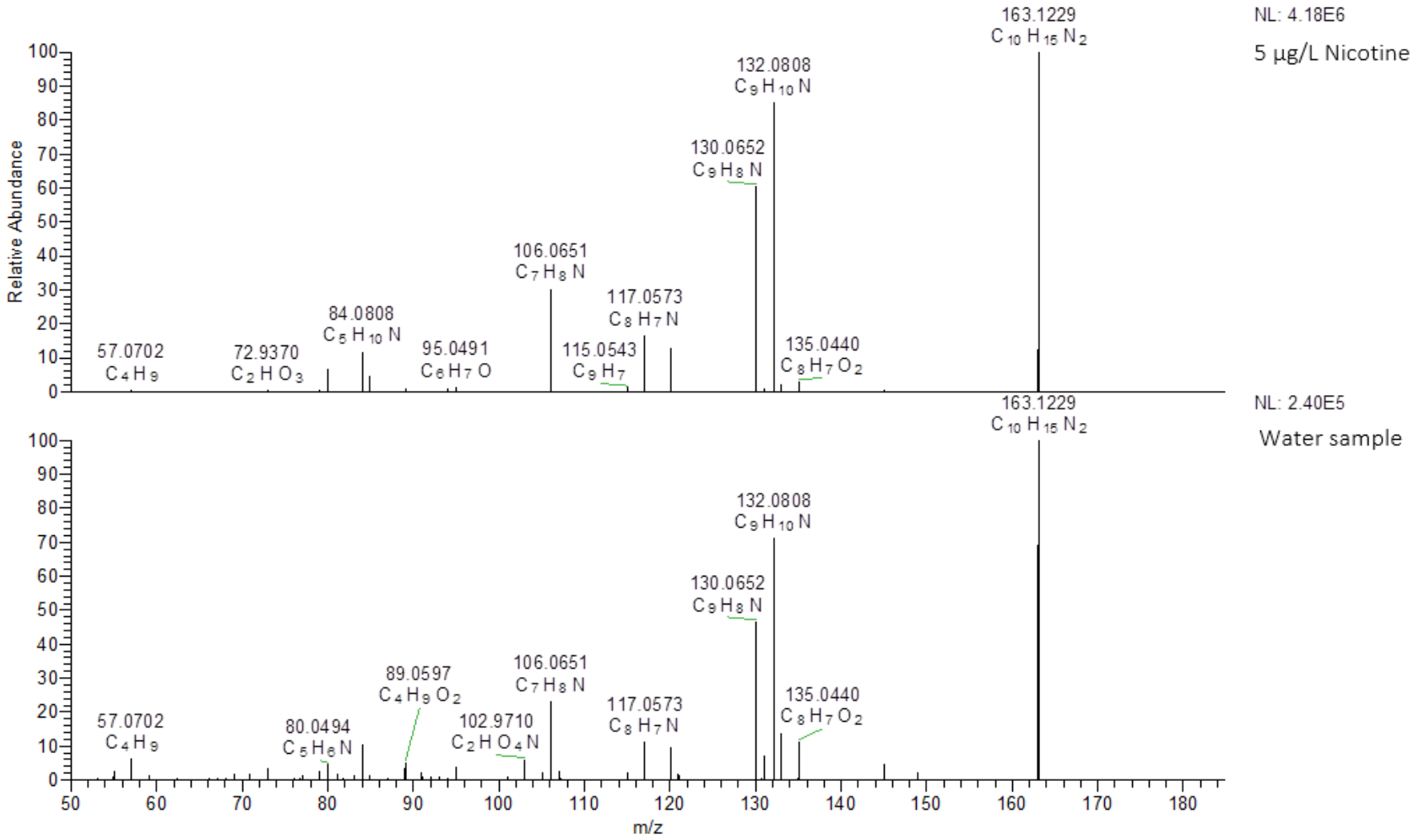

Figure S36: MS/MS spectra (HCD fragmentation at 45 a.u.) of nicotine in a reference standard and water sample (NL: signal intensity at 100% relative abundance).

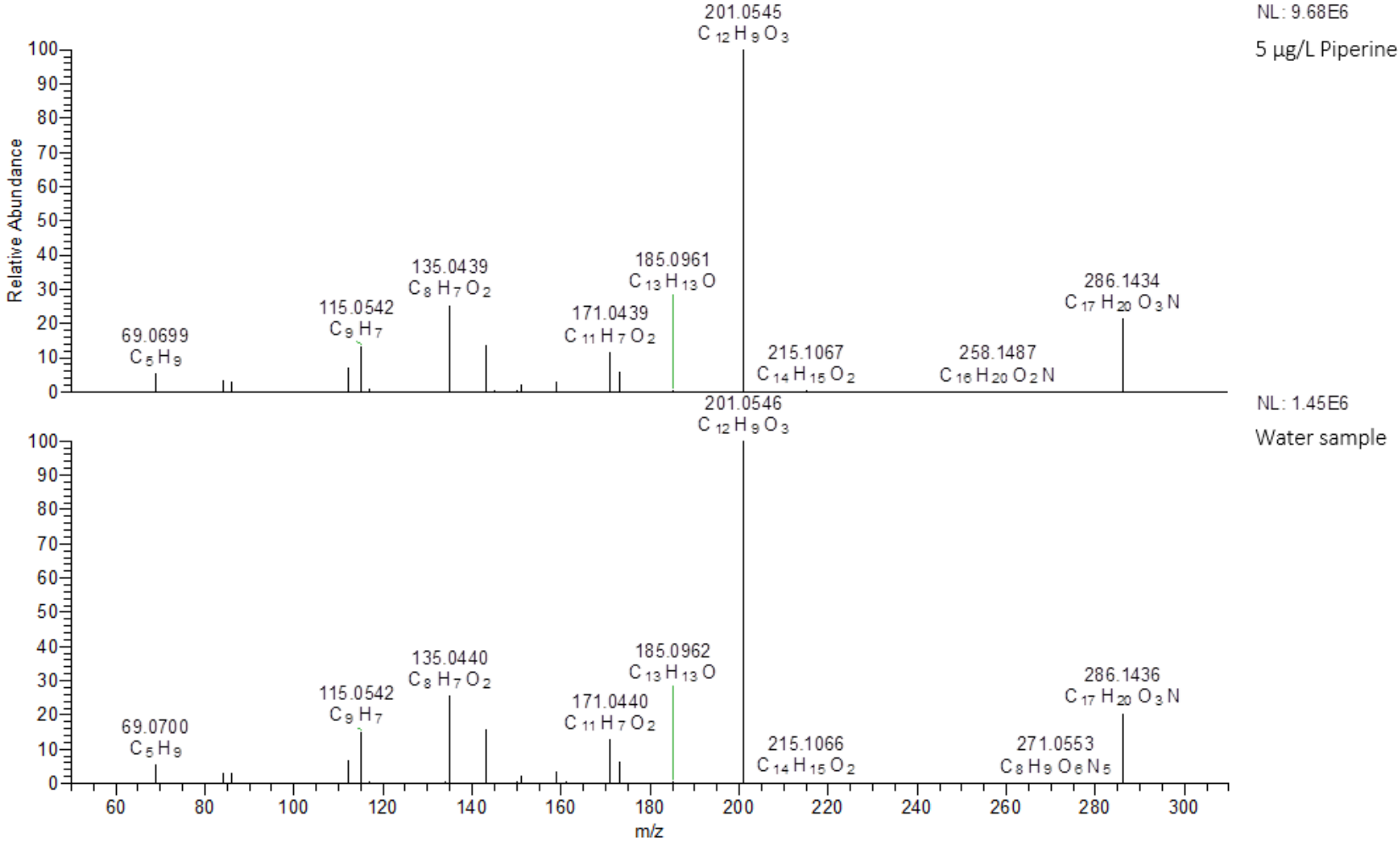

Figure S37: MS/MS spectra (HCD fragmentation at 45 a.u.) of piperine in a reference standard and water sample (NL: signal intensity at 100% relative abundance).

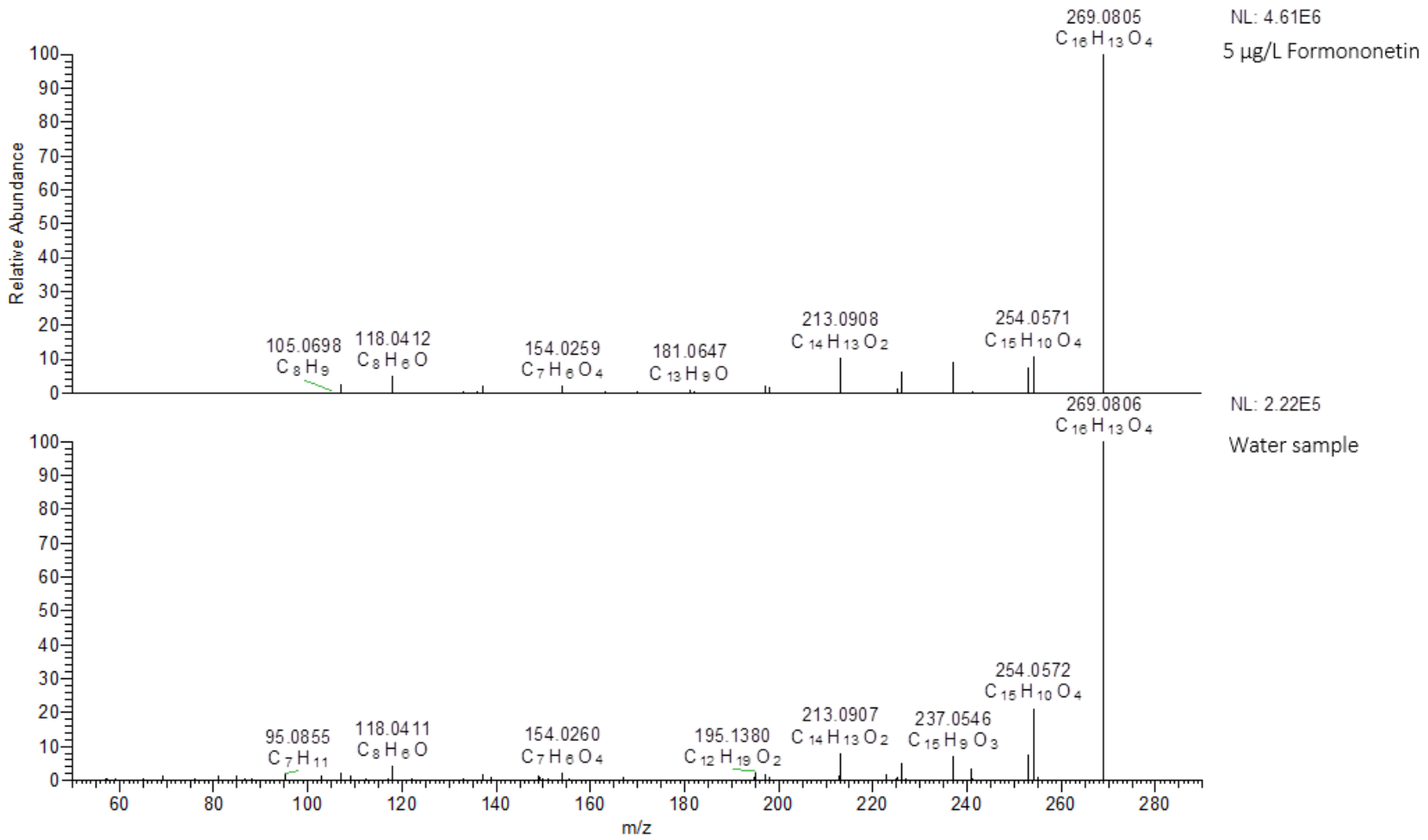

Figure S38: MS/MS spectra (HCD fragmentation at 45 a.u.) of formononetin in a reference standard and water sample (NL: signal intensity at 100% relative abundance).

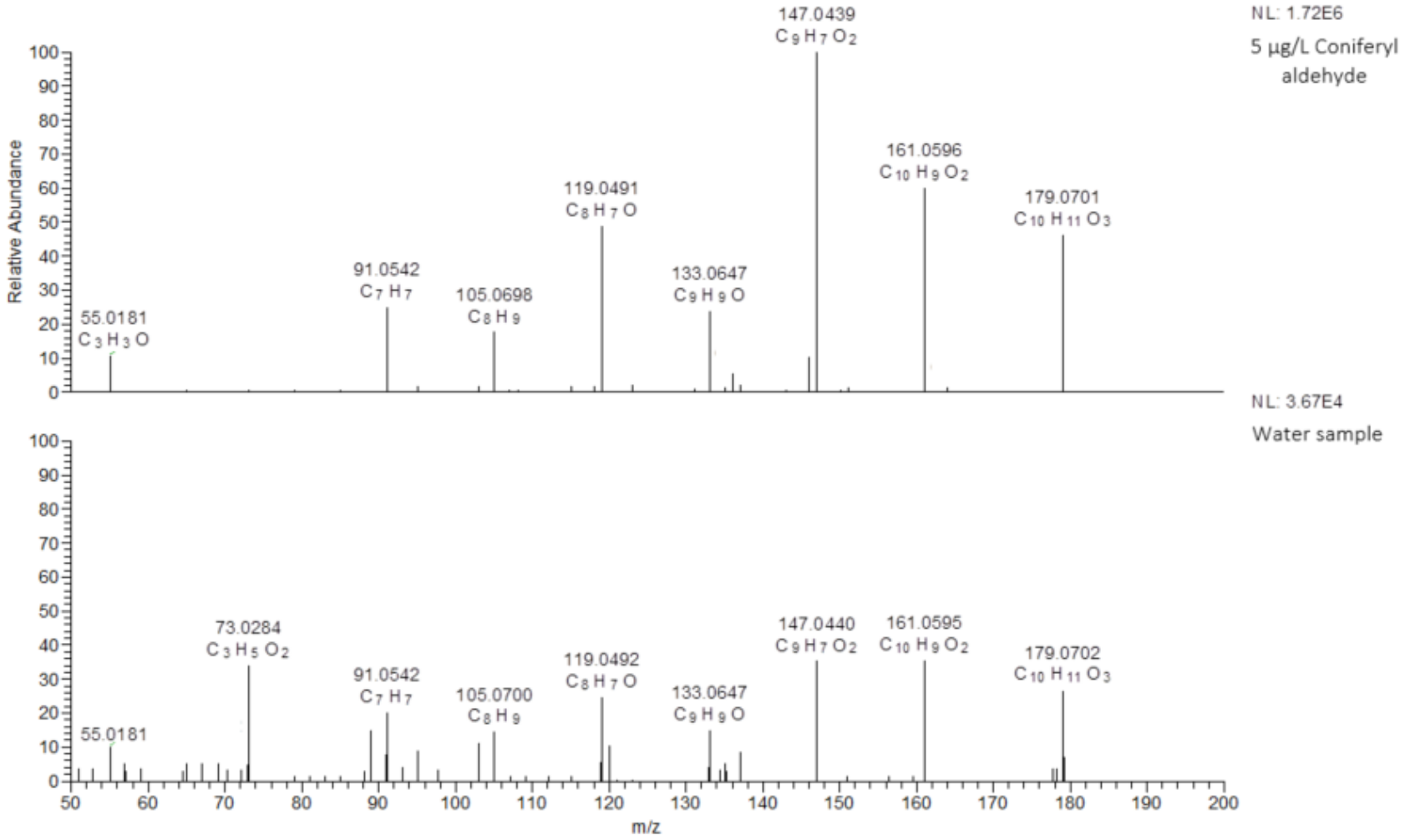

Figure S39: MS/MS spectra (HCD fragmentation at 45 a.u.) of coniferyl aldehyde in a reference standard and water sample (NL: signal intensity at 100% relative abundance).

A.3 Occurrence of emerging contaminants of plant origin in river waters from Vejle, Denmark

Nanusha MY, Krauss M, Sorensen BG, Schulze T, Strobel BW and Brack W

(Submitted manuscript)

Author's contribution statement

Declaration of author contributions to the publication:
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Status: Submitted

Contributing authors: Nanusha MY (1), Krauss M (2), Soerensen BG (3), Schulze T (4), Strobel BW (5) and Brack W (6)

What are the contributions of the doctoral candidate and his co-authors?

1) Concept and design

- ✓ Doctoral candidate: 65 %
- ✓ Co-author 6: 30 %
- ✓ Co-author 2,5: 5 %

2) Conducting tests and experiments

- ✓ Doctoral candidate: 90 %
- ✓ Co-author 2,6: 10 %

3) Compilation of data sets and figures

- ✓ Doctoral candidate: 90 %
- ✓ Co-author 2,6: 10 %

4) Analysis and interpretation of data

- ✓ Doctoral candidate: 90 %
- ✓ Co-author 2,6: 10 %

5) Drafting of manuscript

- ✓ Doctoral candidate: 80 %
- ✓ Co-author 2,6: 15 %
- ✓ Co-author 3, 4 and 5: 5 %

I hereby certify that the information above is correct.

Date and place Signature doctoral candidate

Date and place Supervisor Signature

Occurrence of emerging contaminants of plant origin in river waters from Vejle, Denmark

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Abstract

A large number of chemicals are constantly introduced to surface water from anthropogenic and natural sources. So far, unlike anthropogenic pollutants, naturally occurring compounds are not included in environmental monitoring programs due to limited knowledge on their occurrence and effects in the environment. Since first studies suggest that natural compounds might contribute to mixture risks in aquatic ecosystems and for drinking water production, there is a need to increase empirical evidence on the occurrence of these compounds in aquatic systems. To this end, we performed target screening on 160 toxic secondary plant metabolites (PSMs), prioritized *in silico* for their likelihood of occurrence, persistence, toxicity and mobility in river waters, using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS). The samples were collected during rain events from three Danish rivers from an area covered by grassland, forest and agricultural crops. In total, 27 targets belonging to different compound classes such as alkaloids, coumarins and flavonoids were detected, among them 12 compounds, which have not been reported in surface waters before. The most prominent compound class was the group of alkaloids with 41 % of the detected targets, many of them detected in more than 80 % of the samples. Individual compound concentrations were up to several hundred ng/L with the neurotoxic alkaloid coniine from

poison hemlock and the flavonoid daidzein reaching highest maximum concentrations. Thus, natural toxin concentrations are well within the range of those of synthetic environmental contaminants and need to be considered for the assessment of potential risks on aquatic organisms and drinking water production.

Keywords: Phytotoxins, plant metabolites, surface water, ecotoxicity, natural toxins, emerging contaminants

1. Introduction

A myriad of possibly toxic secondary plant metabolites (PSMs) are synthesized by both natural and agricultural vegetation (Günthardt *et al.* 2018, Hoerger *et al.* 2009a). They are supporting plant's survival and reproductive fitness and function as defense agents (against herbivores, microbes, viruses or competing plants) and signal compounds (to attract pollinating or seed dispersing animals) (Isah 2019, Yang *et al.* 2018). The synthesizing plants release these compounds to the environment as leachate, root exudate and through decomposition of plants (Al-Shatti *et al.* 2014, Aulakh *et al.* 2001). Once PSMs enter to the environment, they often show similar properties as anthropogenic aquatic pollutants in terms of mobility, persistence and possibly also ecotoxicity (Schönsee & Bucheli 2020, Günthardt *et al.* 2020). Structurally, the metabolites belong to different classes of compounds such as pyrrolizidine alkaloids (PAs) including intermedine and echimidine, coumarins including bergapten and psoralen and flavonoids such as quercetin that might impact on aquatic organisms and human health if exposed (Wiedenfeld 2011, Khan *et al.* 2018, Schlatter *et al.* 1991a, Neuman *et al.* 2015, Yang et al. 2018, Al-Shatti et al. 2014). Due to their high toxicity, PAs might be suspected to contribute substantially to toxic risks if wildlife or humans are exposed (Griffiths *et al.* 2020). These compounds are often reported to occur as N-oxides together with their corresponding tertiary alkaloids and are found frequently in some genera of Asteraceae, Boraginaceae and Fabaceae (Ehmke *et al.* 1988). PAs pose genotoxic and carcinogenic risks to animals including humans (Yaber Grass & Leicach 2012, Neuman et al. 2015, Wiedenfeld 2011) and induce liver injury in livestock (Neuman et al. 2015). Flavonoids are widely distributed in a variety of plant species including many edible plants

as dietary components (Miean & Mohamed 2001). Although the majority of natural products are well tolerated, flavonoids and related phytochemicals have been shown to induce neurobehavioral and endocrine disrupting effects. For instance, high doses of quercetin over years have been shown to induce the formation of tumors in mice (Ayaz *et al.* 2019) and may inhibit acetylcholinesterase (AChE) (Ayaz et al. 2019).

Phytochemicals (toxins) have been studied in food and feed for decades, but little attention has been paid to their occurrence in the environment (Hoerger et al. 2009a, Fletcher & Netzel 2020, Jensen *et al.* 2009, Clauson-Kaas *et al.* 2016). Only recently first results on the occurrence of naturally occurring compounds in water and soil have been reported (Hama & Strobel 2019, Hoerger *et al.* 2009b, Günthardt et al. 2020, Hama & Strobel 2020, Nanusha *et al.* 2020a, Nanusha *et al.* 2020b). Despite the large variety of natural compounds that might be leached to surface waters, only few compounds have been reported in surface water. To shed more light on PSMs in the aquatic environment, Nanusha *et al* studied the impact of surrounding vegetation on the chemical mixture in river water using LC-HRMS non-target screening (NTS) and identified overlapping chemicals signal in plant elutriates and potentially impacted river water (Nanusha et al. 2020a). The study revealed thousands of overlapping chemical signals, of which the identites of several compounds such as kaempferol, quercetin and apiin, were confirmed in both water and plants confirming vegetation as source for the occurrence of phytochemicals in river water (Nanusha et al. 2020a). The study also pointed out the impact of rain intensity on the leaching and run-off of phytochemicals into receiving surface waters (Nanusha et al. 2020a). Another study identified the toxic alkaloids lycorine and narciclasine and the photosensitive furanocoumarins bergapten and psoralen in river waters at maximum concentrations of 3 µg/L and 0.5 µg/L, respectively (Nanusha et al. 2020b). The occurrence of estrogenic isoflavones (e.g. formononetin and daidzein), indole alkaloids (e.g. gramine) and pyrrolizidine alkaloids (e.g. senecionine and senkirkine) up to concentrations of 55 ng/L were reported in surface waters from Switzerland (Günthardt et al. 2020, Hoerger et al. 2009b). Hama and Strobel detected pyrrolizidine alkaloids such as jacobine, retrorsine and senecionine in the concentration range of 4 – 270 µg/L in surface water impacted by the high abundance of *Senecio Jacobaea* L. (Hama & Strobel 2019).

We hypothesize that these findings are only the tip of the iceberg and more efforts are needed to explore PSM occurrence in surface water. Thus, in order to extend the knowledge on the impact of PSMs leaching chemical mixtures into surface waters and to understand the impact of abundant (toxic) plants and agriculture on water quality, we selected three connected rivers in Denmark draining a catchment with agricultural land, forest and grassland with high abundance of *Senecio jacobea* to unravel the occurrence of phytotoxins in river water.

2. Materials and Methods

2.1 Water sampling

Our study addressed the Vejle River (Danish: Vejle Å), an approximately 32 kilometre long river, and its two small tributaries (Kvak Moellebaek and Ballegab Skovbaek streams) in Vejle Municipality, Denmark with one sampling site each (Figure 1). Vejle river originates from Engelsholm Lake and flows east through the Vejle River Valley (Danish: Vejle Ådal) until it reaches the City of Vejle. In close vicinity to the sampling sites, the rivers drain agricultural land with barley, wheat and sugarbeet, forest with high abundance of *Alnus glutinosa* (common alder), *Petasites hybridus* (butterbur), *Symphytum x* uplandicum (comfrey), *Urtica dioica* (common nettle) and grassland with *Senecio Jacobaea* L (ragwort).

River water was sampled from October to November 2019, which is a typical rain season. A total of 20 samples of 20 L of water were extracted on-site at the three sites (Table S1 in supplementary information 1 (SI-1)) using large volume solid phase extraction (LVSPE) (Välitalo *et al.* 2017) devices from Maxx Mess-und Probenahmetechnik GmbH, Rangendingen, Germany triggered by the rise in water level resulting from rain events (http://www.hydrometri.dk/hyd/). LVSPE cartridges were filled with 10 g of Chromabond HR–X sorbent (Macherev-Nagel, Düren, Germany) and loaded with extractable components from 20 L of river water per rain event for about 3 hours with a flow rate of approximately 3 mL/min. Extraction cartridges were preconditioned with methanol/ethyl acetate (1:1, v/v), methanol and water. All used solvents had LC-MS grade quality. Loaded cartridges were kept at 4 °C and transported to laboratory. Subsequently,

cartridges were purged with nitrogen to remove water, freeze-dried and stored at -20 °C for analysis. Blanks were prepared in similar manner as samples using the LVSPE device.

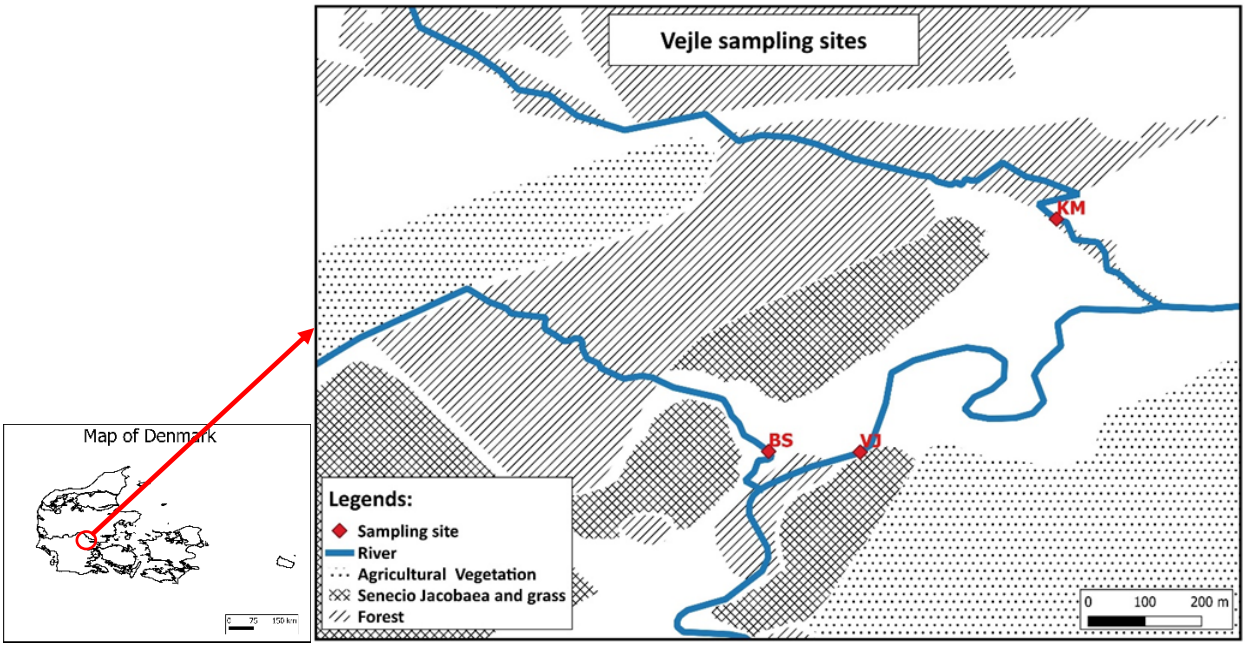


Figure 1: Map showing water sampling sites (Abbreviations: KM – Kvak Moellebaek stream, VJ – Vejle river and BS – Ballegab Skovbaek stream).

2.2 Reagents and chemicals

LC-MS grade methanol, formic acid and ammonium formate were purchased from Honeywell and LC-MS grade water from Thermo-Fisher. LC-MS grade ethyl acetate and 7 N ammonia in methanol were obtained from Sigma-Aldrich. Reference standards were purchased from various suppliers with purity higher than 90 % (see (Nanusha et al. 2020a, Nanusha et al. 2020b) for detailed information).

2.3 Sample preparation

From each cartridge, the analytes were eluted with methanol/ethyl acetate 1:1 (v/v, 500 mL each, neutral fraction), methanol containing 2 % of 7 N ammonia in methanol (500 mL, acidic fraction) and methanol with 1 % of formic acid (500 mL, basic fraction). The pH of both acidic and basic fractions was adjusted to 7 ± 0.5 by adding formic acid or 7 N ammonia in methanol. The eluates were filtered (GF/F Whatman) to remove remaining

precipitates and reduced to dryness using a rotary evaporator (40 °C water bath temperature) and a gentle stream of nitrogen. Subsequently, the samples were transferred to methanol and adjusted to a final enrichment factor of 1000. For analysis, 100-µL aliquots were spiked with 25 µL of internal standard mixture (see Table S2 in SI-1) containing isotope-labelled compounds (1 µg/mL), 30 µL of methanol and 60 µL of water.

2.4 Chemical analysis

For the chemical analysis, 5 µL of the samples were injected into a Thermo Ultimate 3000 LC system (consisting of a ternary pump, autosampler and column oven) coupled to a quadrupole-orbitrap instrument (Thermo QExactive Plus) equipped with a heated electrospray ionisation (ESI) source. Blanks were treated and analysed exactly in the same way as water samples.

Liquid Chromatography. LC separation was performed on a Kinetex C18 EVO column (50 × 2.1 mm, 2.6 µm particle size) using a gradient elution with 0.1 % of formic acid (eluent A) and methanol containing 0.1 % of formic acid (eluent B) at a flow rate of 300 µL/min. After 1 min elution with 5 % B, the fraction of B was linearly increased to 100 % within 12 min and 100 % B were kept for 11 min. Subsequently, the column was rinsed with a mixture of isopropanol + acetone 50:50 / eluent B / eluent A (85 % / 10 % / 5 %) to remove hydrophobic matrix constituents from the column. Finally, the column was re-equilibrated to initial conditions for 5.7 min. The column was operated at 40 °C.

Mass spectrometry. The heated ESI source and the transfer capillary were both operated at 300 °C, with a spray voltage of 3.8 kV, a sheath gas flow rate of 45 a.u. and an auxiliary gas flow rate of 1 a.u. The full scan MS1 was recorded in m/z range 100-1500 with a nominal resolving power of 140,000 (referenced to m/z 200). For metabolite confirmation, data dependent MS/MS acquisition was performed at a resolving power of 70,000 in additional runs. The MS was calibrated externally every two days using the calibration mixtures of the vendor. The mass accuracy was always within 5 ppm for all analyses. All MS and MS/MS analyses were performed in ESI positive (ESIpos) and negative (ESIneg) mode.

2.5 Target screening

2.5.1 Qualitative target screening

About 160 target compounds have been prioritized for their likelihood to occur in surface waters, their toxicity and commercial availability for screening of water samples as described by (Nanusha et al. 2020a, Nanusha et al. 2020b). The LC-HRMS raw data were converted to mzML format using ProteoWizard (version 2.1.0) (Holman et al. 2014). The centroid data were subjected to MZmine (version 2.38) for peak detection followed by peak alignment and target compound annotation (Müller *et al.* 2020, Katajamaa & Oresic 2005, Pluskal *et al.* 2010). Settings for each step of the data processing are given in SI-2 (Table S3). Further evaluation and visualization were performed using Excel 2013 (Microsoft office) and R (version 3.4.3).

Target compounds were identified by matching m/z and retention time between water samples and standard compounds with a mass and retention time tolerance of 5 ppm and +/- 0.1 min, respectively. In order to exclude noise and background signals the cut-off intensity was set to 10^4 and data were corrected for blank signals based on seven blanks analyzed together with the samples. Duplicates resulting from multiple annotation were removed manually using peak resolution and intensity. For the tentatively identified target compounds, an inclusion list was developed for data dependent MS/MS acquisition. MS/MS experiments were conducted on authentic standard compounds and the samples to confirm the chemical structure. Diagnostic MS/MS fragments were matched with the MS/MS of reference standards. For the target compounds with low intensity in unresolved chromatograms, parallel reaction monitoring analysis was conducted for better chromatographic peaks visualization. The XCalibur v4.0.27.10 (Thermo Fisher Scientific) software was used for analysis of extracted ion chromatograms (EICs) and mass spectra (MS1 and MS2).

2.5.2 Quantification of detected targets

TraceFinder 4.1 (ThermoFisher Scientific) was used for the quantification of the confirmed target compounds using extracted ion chromatograms of the full scan data. In TraceFinder, the use of only one identifier mass (precursor ion) bares the risk of false

positive identification and quantification of contaminants. Thus, additional fragment ions were used to confirm the presence of target compounds and to eliminate errors in identification. For some metabolites, ions used for confirmation were not clearly detectable due to low intensity. In such cases, confirmation was complemented using Xcalibur. A series of method-matched calibration standards ranging from 0.5 to 5000 ng/L were used. All the calibration standards were treated exactly the same way as river water samples. The target compounds were quantified using the internal standards with the nearest retention time. The method detection limits (MDLs) (Table 1) for the detected target compounds were determined following US-EPA procedure (US-EPA 2011).

3. Results and discussion

3.1 Occurrence of target compounds in river waters

In total, 226 peaks were detected with an agreement of the precursor ion mass (m/z) and retention time with target compounds at mass and retention time tolerance of 5 ppm and +/- 0.1 min, respectively. Some target compounds were annotated several times due to picking multiple peaks at a single precursor ion mass with given retention time tolerance or due to their different adducts ($M+H^+$, $M+NH_4^+$ and $M+Na^+$). Removal of duplicates and false positives and peak filtering for intensity and resolution reduced the target list to 138 annotated peaks. Based on additional MS/MS fragment comparison with reference standards, we confirmed the presence of 27 target compounds in all samples from three rivers. The detected compounds represent a wide variety of natural compounds that belong to different compound classes such as alkaloids, coumarins, flavonoids and others, with alkaloids being the prominent compound class. The chemical structures for those compounds not reported previously (Nanusha et al. 2020a, Nanusha et al. 2020b) are given in Figure 2. Identified compounds include the alkaloids coniine, cytisin and intermedine and the coumarins psoralen and fraxetin. The details on the identified target compounds are given in Table S4 in SI-2. The samples are named according to the river name, i.e. VJ denotes samples collected from Vejle River, KM denotes Kvak Moellebaek Stream and BS denotes Ballegab Skovbaek Stream.

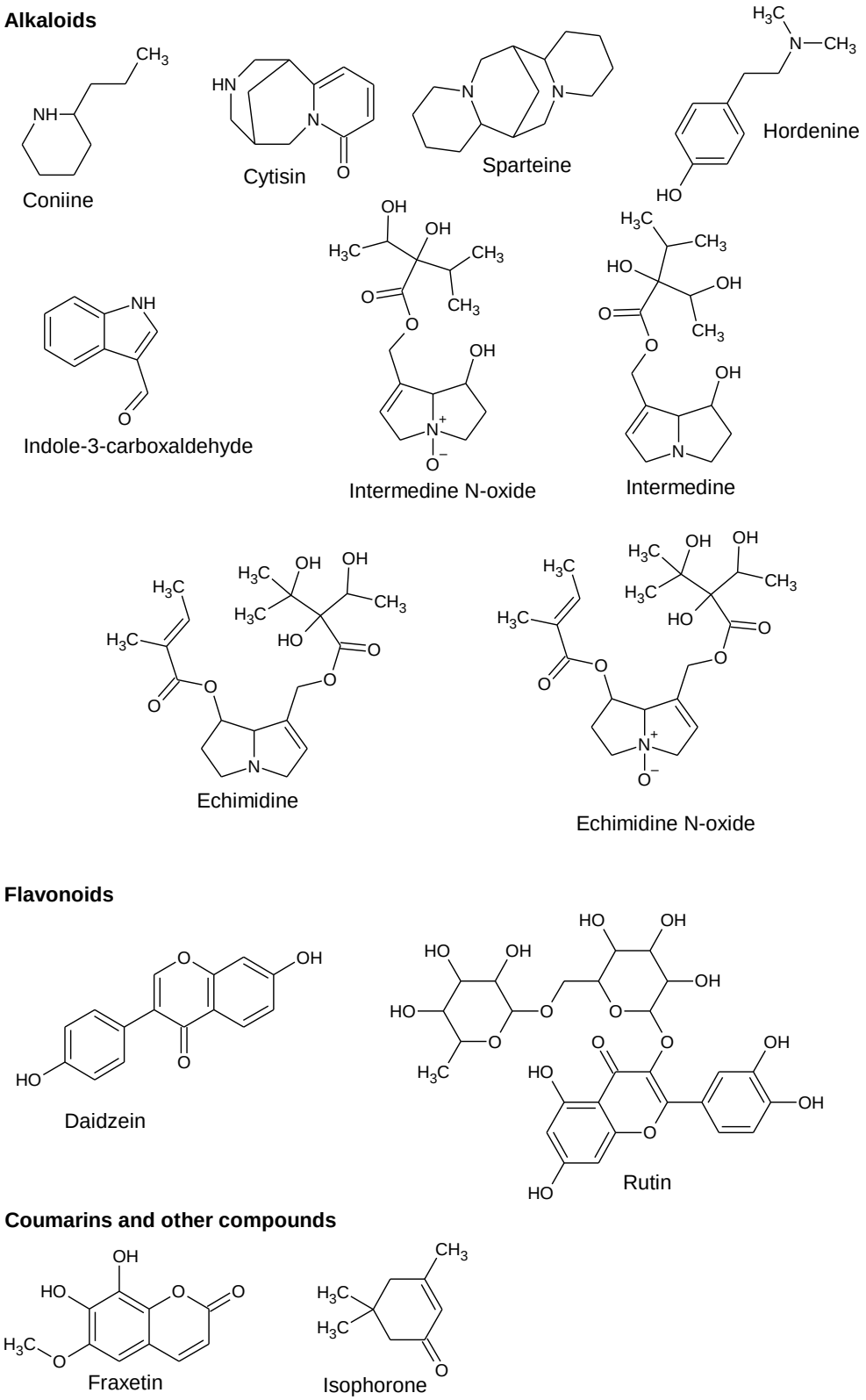


Figure 2: Chemical structure for some of the detected target compounds (not reported previously by (Nanusha et al. 2020a, Nanusha et al. 2020b)).

All water samples contained at least 13 co-occurring targets with a maximum of 20 targets in one sample from Kvak Moellebaek stream. Detection frequency of individual targets ranged from 5 % (detection in only one sample) for psoralen and lycopsamine N-oxide to 100 % for fraxidin, indole3-carboxaldehyde, intermedine N-oxide, isofraxidin and scopoletin (Figure 3). Among the identified targets, 48 % were detected in more than 80 % of the samples while 19 % (5 metabolites) were detected in all samples from the three rivers. The good agreement between the sampling sites is linked with the similar land use, vegetation type and density in the catchment although the detection frequency of the targets was slightly higher in Vejle river than in the streams Kvak Moellebaek and Ballegap Skovbaek.

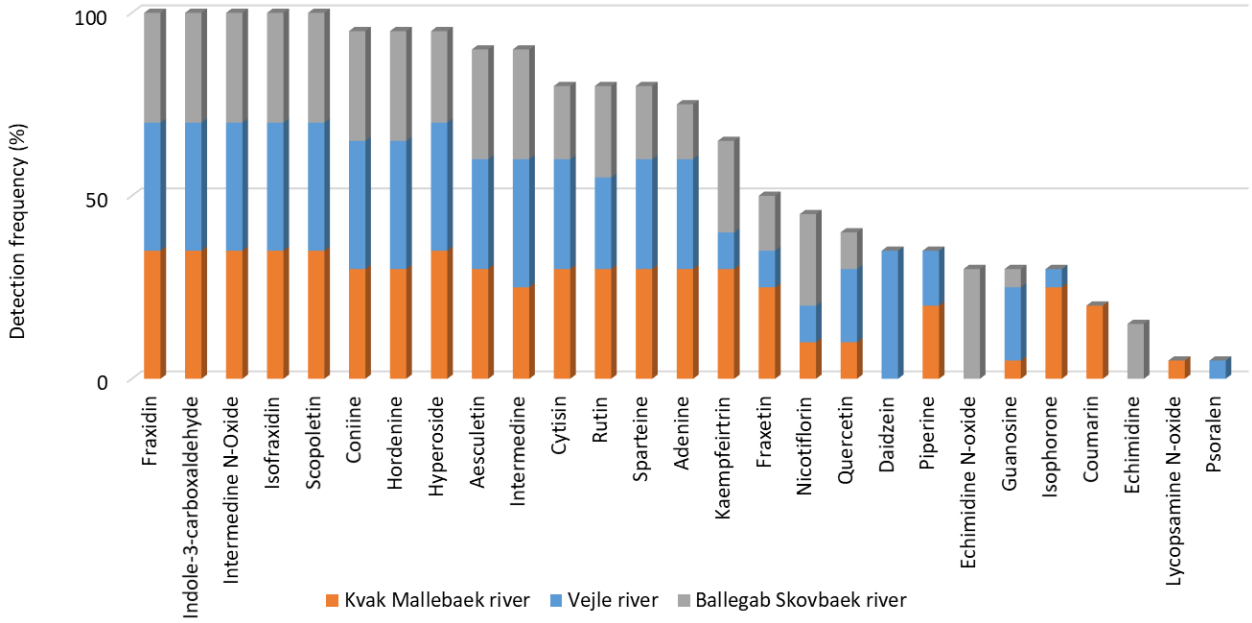


Figure 3: The detection frequency of the identified target compounds.

3.2 Concentration of target compounds

Table 1 summarizes the concentration range of target compounds detected in samples from the three studied rivers, while the individual concentrations are given in Table S4 in SI-2. Individual concentrations ranged from 0.4 to 191, from 0.3 to 400 and from 0.5 to 62 ng/L in samples from Kvak Moellebaek, Vejle and Ballegab Skovbaek rivers, respectively. Some target compounds were obtained in samples from one site only such as daidzein and psoralen in Vejle river, lycopsamine N-oxide in Kvak Moellebaek stream and

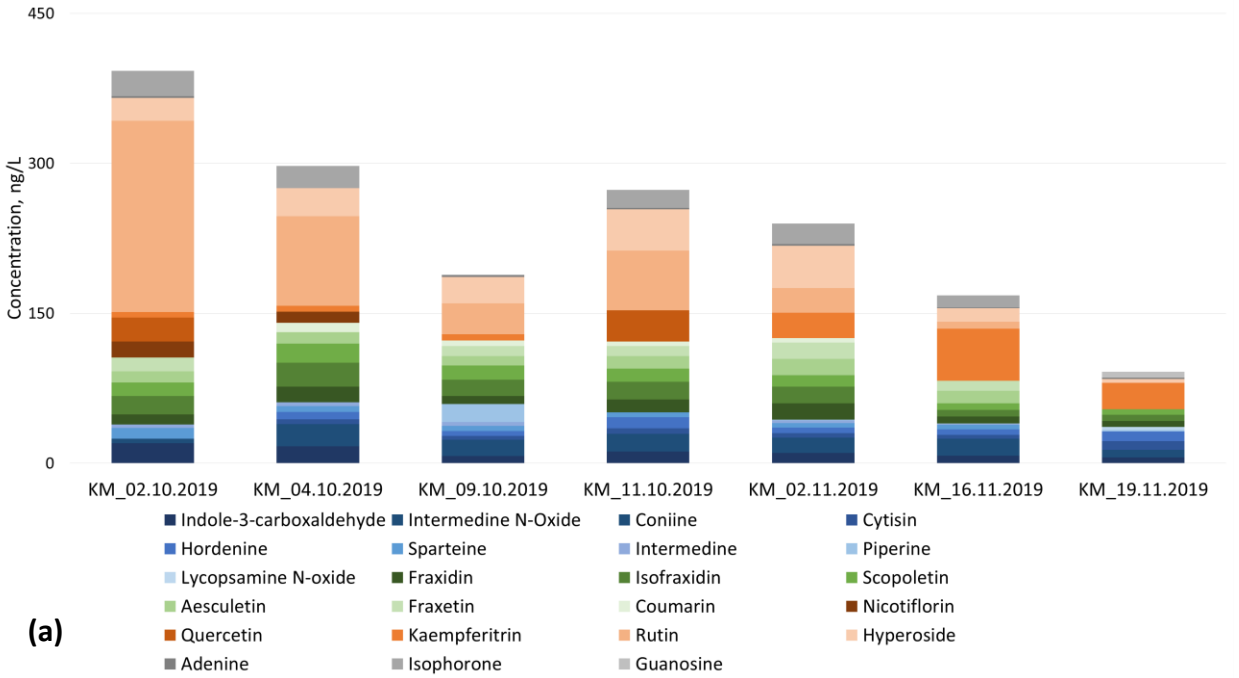
coumarin and echimidine N-oxide in Ballegab Skovbaek stream. For many of the detected targets, Vejle river contained highest individual concentrations. This finding is in agreement with the existence of several upstream tributaries contributing to the load. Out of 27 detected compounds, 11 (41 %) were alkaloids with individual concentration ranging from 1 to 400 ng/L, followed by coumarins (26 % of detected targets and a concentrations range of 3.7 to 191 ng/L) and flavonoids (22 % of the detected target compounds and concentrations from 4.5 to 49 ng/L).

Table 1: The concentration rage (min – max, ng/L) of individual targets identified in water samples from three rivers (Abbreviations: KM – Kvak Moellebaek; VJ – river; BS – Ballegab Skovbaek stream; ND- Not detected; MDL – method detection limit)

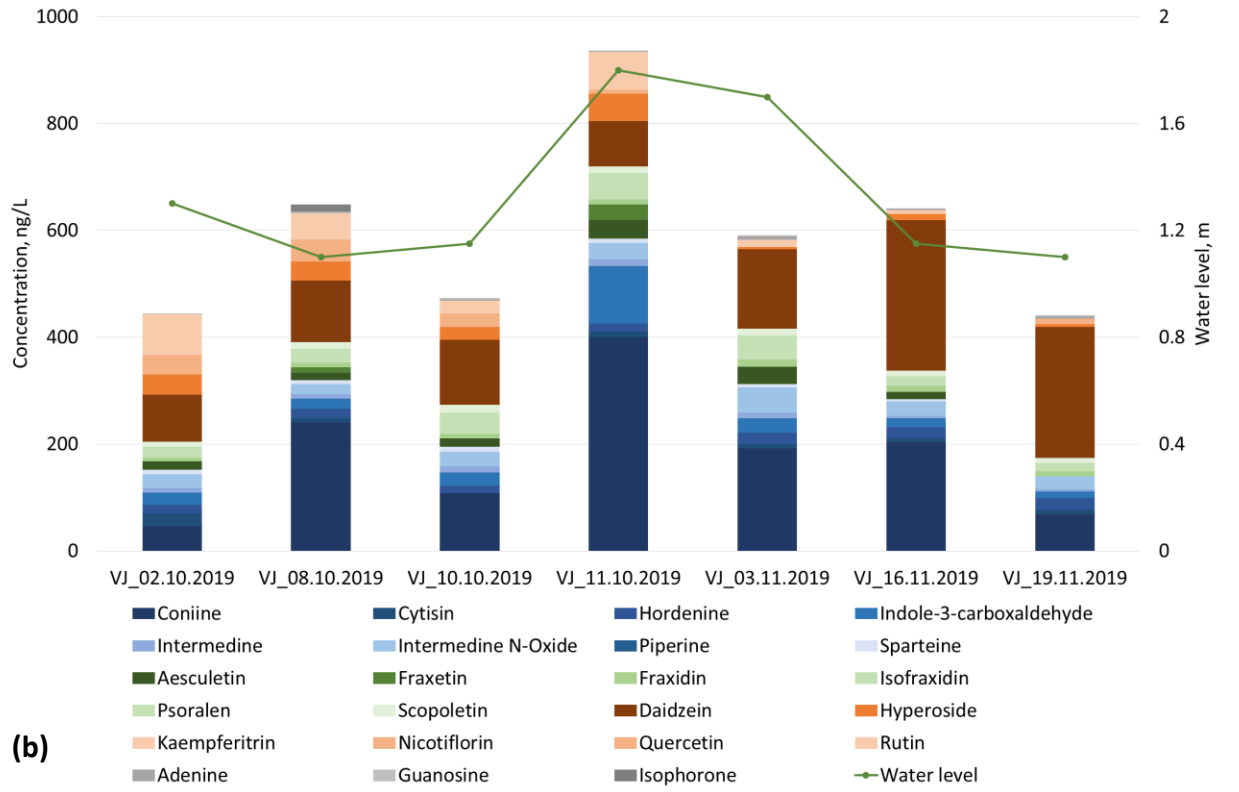
Compound name	Compound class	Formula	CAS No	m/z	RT (min)	MDL, ng/L	Concentration range (min - max, ng/L)		
							BS	KM	VJ
Adenine	Purine base	$C_5H_5N_5$	73-24-5	136.0619	0.5	0.5	0.5 - 2.4	0.7 - 1.9	1.6 - 6.1
Aesculetin	Coumarin	$C_9H_6O_4$	305-01-1	179.0336	3.6	5.0	7 - 22.6	9.4 - 16.4	13.2 - 34.8
Coniine	Alkaloid	$C_8H_{17}N$	458-88-8	128.1433	7.1	3.0	3.8 - 8.9	3.3 - 8.7	45.7 - 400.5
Coumarin	Coumarin	$C_9H_6O_2$	91-64-5	147.0441	7.3	3.6	ND	4.5 - 9.5	ND
Cytisin	Alkaloid	$C_{11}H_{14}N_2O$	485-35-8	191.1179	0.5	1.0	4.6-8	3.8 - 8.8	7.6 - 24.8
Daidzein	Flavonoid	$C_{15}H_{10}O_4$	486-66-8	255.065	9.5	1.0	ND	ND	84.7 - 281.9
Echimidine	Alkaloid	$C2_0H_{31}NO_7$	520-68-3	398.217	6.4	0.8	3.4 - 4.2	ND	ND
Echimidine N-oxide	Alkaloid	$C_{20}H_{31}NO_8$	41093-89-4	414.2117	6.5	2.7	13.5 - 34.7	ND	ND
Fraxetin	Coumarin	$C_{10}H_8O_5$	574-84-5	209.0443	6.2	3.5	11 - 20.3	10 - 16.3	11 - 29.3
Fraxidin	Coumarin	$C_{11}H1_0O_5$	525-21-3	223.06	7.8	1.8	4.9 - 15.4	5.9 - 16.3	7 - 13.8
Guanosine	Purine base	$C_{10}H_{13}N_5O_5$	118-00-3	284.0984	0.5	1.5	2.4	5.8	2.5 - 2.8
Hordenine	Alkaloid	$C_{10}H_{15}NO$	539-15-1	166.1226	0.5	1.6	5.5 - 11.5	5 - 11.3	13.1 - 21.9
Hyperoside	Flavonoid	$C_{21}H_{20}O_{12}$	482-36-0	465.1017	8.7	2.7	5.5 - 36.2	3.9 - 42.6	3.7 - 51.6
Indole-3-carboxaldehyde	Alkaloid	C_9H_7NO	487-89-8	146.0601	6.8	3.0	5.3 - 18.2	5.9 - 20.1	12.2 - 108.5
Intermedine	Alkaloid	$C_{15}H_{25}NO_5$	10285-06-0	300.1801	0.7	0.5	1.3 - 8.5	1.2 - 3.9	4.2 - 12.5
Intermedine N-oxide	Alkaloid	$C_{15}H_{25}NO_6$	95462-14-9	316.1752	0.8	1.3	11.5 - 24.8	4.2 - 15.8	18.9 - 47
Isofraxidin	Coumarin	$C_{11}H_{10}O_5$	486-21-5	223.0599	7.4	3.9	5.9 - 30.1	6.3 - 24	15.2 - 49.1
Isophorone	Cyclic ketone	$C_9H_{14}O$	78-59-1	139.1117	9.0	5.5	ND	12.2 - 25.1	13.6
Kaempferitrin	Flavonoid	$C_{27}H_{30}O_{14}$	482-38-2	579.1707	9.3	4.3	5.5 - 19.4	5.8 - 51.8	5.4 - 6.8
Lycopsamine N-oxide	Alkaloid	$C_{15}H_{25}NO_6$	95462-15-0	316.1751	0.5	0.5	ND	3.2	ND
Nicotiflorin	Flavonoid	$C_{27}H_{30}O_{15}$	17650-84-9	595.165	9.3	3.0	5.2 - 26.4	11.2 - 15.9	6.6 - 7.2
Piperine	Alkaloid	$C_{17}H_{19}NO_3$	94-62-2	286.1434	12	0.2	ND	0.4 - 18.1	0.3 - 0.4
Psoralen	Coumarin	$C_{11}H_6O_3$	66-97-7	187.0388	9.2	2.9	ND	ND	5.0
Quercetin	Flavonoid	$C_{15}H_{10}O_7$	117-39-5	303.0496	8.6	5.3	23 - 23.5	23.9 - 31.2	11.3 - 36.5
Rutin	Flavonoid	$C_{27}H_{30}O_{16}$	153-18-4	611.1604	8.7	5.0	5.2 - 62	7.2 - 191	8.2 - 76.3
Scopoletin	Coumarin	$C_{10}H_8O_4$	92-61-5	193.0496	7.0	1.5	7.7 - 22.6	5.5 - 19.1	9.1 - 14
Sparteine	Alkaloid	$C_{15}H_{26}N_2$	90-39-1	235.2168	0.7	1.7	4.5 - 6.8	4.4 - 10.8	4.5 - 9.7

3.3 Dependence of concentrations on raise in water level

Figure 4 demonstrates concentration trends for target compounds obtained per river over time reflecting raising water levels and thus rain intensity. Since sampling was triggered by raise in water level due to rain events, the sampling time courses are different for the three rivers with more similarity between Kvak Moellebaek and Vejle rivers (Figure 4 a and b). In Figure 4, blue color stands for alkaloids, green for coumarins, red for flavonoids and grey for other miscellaneous compounds. Typically, maximum concentrations were obtained in October samples reflecting high rain intensity and probably higher activity of the plants than in November (Isah 2019). In general, the overall trend of change in concentration seems consistent with the rise in water level for Vejle river while concentrations decreased in the other two streams. In samples from Kvak Moellebaek stream (Figure 4a), the differences in total concentration of targets were mainly driven by flavonoids, specifically by rutin, while the contribution from coumarins, alkaloids and other miscellaneous compounds remain relatively constant throughout the samples, except in the last sample (KM_19.11.2019). Regarding samples from Vejle river (Figure 4b), the variation in overall concentration was mainly driven by the alkaloid coniine and the flavonoid daidzein behaving in opposite way throughout the samples. Lower water levels tend to increase daidzein concentrations while higher water levels increase coniine concentrations. This agrees with the hypothesis that daidzein does not come from plant leachate but from domestic waste due to soya product consumption in households and diluted at high water levels, while coniine behaves as expected for a compound leaching from vegetation during rain. Samples from Ballegab Skovbaek stream (Figure 4c) showed different behavior of concentrations of target compounds with continuous decline over time, from October to November samples.


450
300
150
0
Concentration, ng/L
KM_02.10.2019
KM_04.10.2019
KM_09.10.2019
KM_11.10.2019
KM_02.11.2019
KM_16.11.2019
KM_19.11.2019
Indole-3-carboxaldehyde
Intermedine N-Oxide
Coniine
Cytisin
Hordenine
Sparteine
Intermedine
Piperine
Lycopsamine N-oxide
Fraxidin
Isofraxidin
Scopoletin
Aesculetin
Fraxetin
Coumarin
Nicotiflorin
Quercetin
Kaempferitrin
Rutin
Hyperoside
Adenine
Isophorone
Guanosine

(a)


1000
800
600
400
200
0
2
1.6
1.2
0.8
0.4
0
Concentration, ng/L
Water level, m
VJ_02.10.2019
VJ_08.10.2019
VJ_10.10.2019
VJ_11.10.2019
VJ_03.11.2019
VJ_16.11.2019
VJ_19.11.2019
Coniine
Cytisin
Hordenine
Indole-3-carboxaldehyde
Intermedine
Intermedine N-Oxide
Piperine
Sparteine
Aesculetin
Fraxetin
Fraxidin
Isofraxidin
Psoralen
Scopoletin
Daidzein
Hyperoside
Kaempferitrin
Nicotiflorin
Quercetin
Rutin
Adenine
Guanosine
Isophorone
Water level

(b)

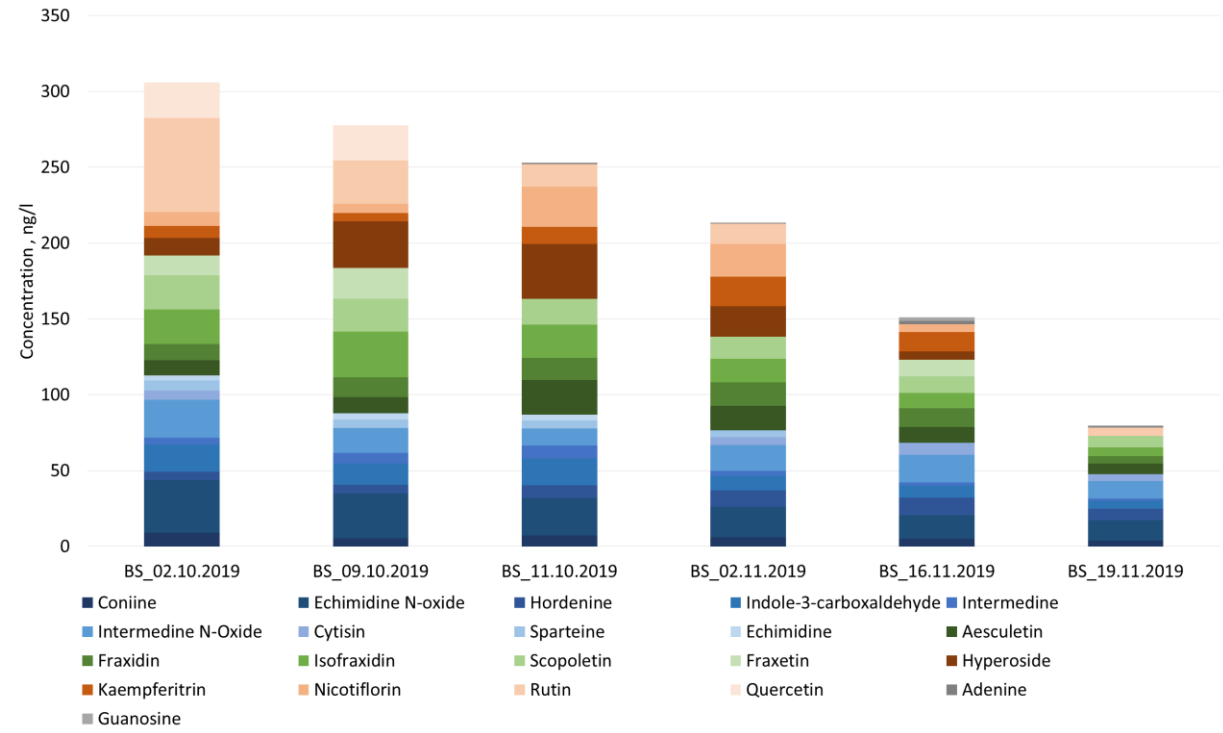


Figure 4: Showing sampling date or rain intensity dependent variation in concentration of target compounds identified in water samples per river; (a) KM – Kvak Moellebaek stream, (b) VJ – Vejle river and (c) BS – Ballegab Skovbaek stream; Sample ID: river name_sampling or rain event date.

3.4 Distribution of individual targets

3.4.1 Alkaloids

The PAs intermedine and intermedine N-oxide were obtained at detection frequencies of 90 and 100 % and in concentrations up to 12.5 and 47 ng/L, respectively. Other PAs, echimidine, echimidine N-oxide and lycopsamine N-oxide were obtained at lower detection frequency (less than 30 % of the samples) at concentrations of up to 34.7 ng/L. The compounds were previously reported in different Boraginaceae including *Symphytum bulbosum*, *Symphytum officinale* and *Symphytum tuberosum* (Günthardt et al. 2018, Brauchli *et al.* 1982, Brown *et al.* 2016, Salehi *et al.* 2019, Mei *et al.* 2010). This is in agreement with the abundant presence of *Symphytum x uplandicum* along Ballegab Skovbaek and Vejle rivers. Lycopsamine and intermedine extracted from *Symphytum officinale* were demonstrated to cause adverse effects such as angiectasis at a concentration of 1500 mg/kg in rats. Similar effects on chicken liver were reported at a

concentration of 77 mg/kg (Brown et al. 2016). Although, in the environment, these compounds occur at levels that are too low to produce acute liver damage, they are still high enough to be of concern as a possible long-term cause of cirrhosis and liver failure in organisms (van Egmond 2004). Interestingly the PAs senecionine, jacobine, erucifoline and seneciphylline that are known to occur in *Senecio jacobea* have not been detected in the water samples despite the high abundance of this plant (Hama & Strobel 2020, Hama & Strobel 2019) .

The quinolizidine alkaloids, cytisin and sparteine, were detected in 80 % of samples from the three rivers. The former was found at an average concentration of 3.8, 4.5 and 9.5 ng/L while the latter reached 3.2, 5.1 and 6.3 ng/L in samples from Ballegab Skovbaek, Kvak Moellebaek and Vejle rivers, respectively (Table 1). The compounds were identified as the main alkaloids from *Cytisus scoparius* (common broom), but it can also be isolated from several Fabaceae species, including *Lupinus, Spartium, and Cytisus* (Afendi *et al.* 2012, Günthardt et al. 2018, Rosenmeier *et al.* 2013). Apart from their numerous pharmacological effects, e.g. cardiovascular and antihypertensive, cytisin and sparteine demonstrated inhibitory effect on the central nicotinic acetylcholine receptors at IC_{50} of approximately 26 µg/L and 77 mg/L, respectively, based on *in vitro* studies (Schmeller *et al.* 1994, Villalpando-Vargas & Medina-Ceja 2016).

The phenethylamine alkaloid hordenine was detected in 95 % of samples at an average concentration range of 6.3 to 17.9 ng/L (Figure 3 and Table S4 in SI-2). Most commonly, it is extractable from barley (*Hordeum* species) providing also the name. However, it can be found in a variety of natural and agricultural plants including grasses (Afendi et al. 2012, Hoult & Lovett 1993, Frank *et al.* 1990). Its detection in the three rivers is in agreement with the abundance of agriculture and grass land in the catchment. The compound exhibits numerous pharmacological effects causing respiratory distress in horses at an effect concentration of 2 mg/kg due to its indirect action as adrenergic drug (Kim *et al.* 2013, Hoult & Lovett 1993, Frank et al. 1990). Hordenine may decrease the UV protection by inhibiting the production of melanin, which plays an important role in protecting skin against ultraviolet light injury (Kim et al. 2013).

Coniine, a polyketide-derived alkaloid, was detected in 95 % of samples from three sites (Figure 3). It was detected at an average concentration range of 5.2 to 179.7 ng/L – the highest occurring in samples from Vejle river (Table S4 in SI-2). Coniine is known to occur in toxic Apiaceae such as *Conium maculatum* (Afendi et al. 2012, Günthardt et al. 2018, López *et al.* 1999). However, there was no evidence for the occurrence of such plants alongside of the sampled rivers. It is a nicotinic acetylcholine receptor antagonist inhibiting the nervous system, eventually causing death (López et al. 1999, Hotti & Rischer 2017, Hotti *et al.* 2015). Coniine's most famous victim is Socrates who was sentenced to death by poison chalice containing poison hemlock in 399 BC (Hotti & Rischer 2017). Following the administration of coniine, signs of maternal intoxication were observed in both rat and rabbit (Forsyth & Frank 1993).

Piperine, a piperidine alkaloid, was detected in samples from the rivers Kvak Moellebaek and Vejle in concentrations up to 18.1 ng/L. It was also previously reported in river waters from Germany at concentration up to 338 ng/L (Nanusha et al. 2020b). Piperine is a major component of Piper species (e.g. *Piper nigrum, Piper longum, Piper officinarum and Piper retrofractum*), which are globally marketed as flavoring agent and cooking spice with a long history of human health benefits and a wide consumption (Shoba *et al.* 1998, Schnabel *et al.* 2020). Thus, the input of piperine to the river water is very likely due to human activities, while no plants containing these compounds in the catchments are known. Besides its numerous medicinal benefits such as antioxidant, antithyroid and antiasthmatic activity, piperine may also have adverse effects including hemorrhagic necrosis and edema in gastrointestinal tract, urinary bladder and adrenal glands observed in animal tests with rats (Derosa *et al.* 2016, Piyachaturawat *et al.* 1983). Zwart *et al* detected piperine in waste water treatment plant effluents and classified it as one of the most potent nonsteroidal estrogens (Zwart *et al.* 2018).

Indole-3-carboxaldehyde, an indole alkaloid, was detected in all samples from all three rivers (100 % detection frequency – Figure 3). It was quantified within the concentration range of 5.3 – 108.5 ng/L in samples, the maximum concentration was measured in Vejle river. It's extractable from several plants such as barley (*Hordeum vulgare*) (Puri *et al.* 1998, Afendi et al. 2012)

3.4.2 Flavonoids

Daidzein was detected in all samples from Vejle river, only, up to a maximum concentration of 281.9 ng/L. It was previously reported in creeks from Switzerland up to a concentration of 5.5 ng/L (Günthardt et al. 2020), up to 40 ng/L in rivers in Iowa (Kolpin *et al.* 2010), while concentrations in the River Danube downstream of untreated wastewater discharge reached almost 500 ng/L (König *et al.* 2017). Daidzein, whose chemical structure is a naturally occurring isoflavonoid phytoestrogen belonging to the non-steroidal estrogens, is mainly derived from the Fabaceae family plants such as soybean, peas and red clover (Ayaz et al. 2019, Liu *et al.* 2007, Hoerger et al. 2009b, Afendi et al. 2012). Through its way from the origin, Vejle river flows long distance (approximately 32 Km) and passes through various farmland, which may contain such plants as the origin of the compound. Alternatively, its detection in river could likely be associated with human activities, since leguminous plants are widely used as sources of food. This hypothesis is supported by the fact that higher water levels in Vejle River are accompanied by lower daidzein concentrations supporting dilution of municipal wastewater rather than leaching from vegetation as driver of concentration changes. Daidzein was investigated for its potential to alter fertility and cause developmental toxicity to the reproductive tract in female rats and has been reported to affect various neurobiological regulatory mechanisms such as behavior, cognition, growth, development and reproduction (Lamartiniere *et al.* 2002, Ahmed *et al.* 2017).

Rutin and hyperoside, both glycosides of the flavonoid quercetin, were obtained with a detection frequency of > 80 % (Figure 3) and concentrations up to 190.9 ng/L (Table 1). Their aglycone quercetin was also detected in 40 % of samples at concertations of 11.3 to 36.5 ng/L. Hyperoside is a typical component of *Hypericum perforatum*, quercetin from *Quercus* (oak) while rutin is synthesized by both plants (Afendi et al. 2012) as well as by *Symphytum officinale* (Tahirovic et al. 2010). The detection of rutin at high concentration (190.9 ng/L), among flavonoids, could be linked to the abundant presence of *Symphytum x uplandicum*. The occurrence of quercetin and rutin could also be connected with the high abundance of *Urtica dioica* along the rivers (Afendi et al. 2012). Recently, hyperoside and quercetin were reported in river water as well as in extracts of *Galanthus nivalis* and

Fraxinus excelsior abundantly present in close vicinity (upstream) to the water sampling sites, confirming that the occurrence of these compounds in river water is linked to the surrounding vegetation (Nanusha et al. 2020a). The authors found quercetin and hyperoside in river waters at considerable concentrations up to 2.5 and 4 µg/L, respectively (Nanusha et al. 2020a). Vila-Nova and colleagues isolated the flavonoids quercetin and rutin from *Dimorphandra gardneriana* and *Platymiscium floribundum* and *in vitro* assay determined acetylcholinesterase enzyme (AChE) inhibition with EC_{50} of 10.6 and 43.3 µg/mL, respectively (Vila-Nova *et al.* 2012). Numerous pharmacological applications were reported for hyperoside, for example for the improvement of the cardiac function and for the treatment of liver fibrosis (Wang *et al.* 2016) (Wang *et al.* 2018). The same compound displayed acetylcholinesterase inhibition and depression of the central nervous system (Hernandez *et al.* 2010, Haas *et al.* 2011).

The kaempferol glycosides nicotiflorin and kaempferitrin were detected in samples from all three rivers with a detection frequency of 65 and 45 %, and maximum concentrations of 26.4 ng/L and 51.8 ng/L, respectively (Table 1). Nicotiflorin and kaempferitrin were previously reported in river water from Germany at maximum concentrations of approximately 2 and 1 µg/L, respectively (Nanusha et al. 2020a). Nicotiflorin is synthesized by *Urtica dioica* (Afendi et al. 2012). Both compounds decrease arterial blood pressure and heart beat rate and have hepatoprotective effects (Harborne & Baxter 1999). Nicotiflorin was found to protect against memory dysfunction and oxidative stress in multi-infarct dementia model rats (Huang *et al.* 2007, Harborne & Baxter 1999). A study by Zhang *et al* showed that kaempferitrin competitively inhibited human liver microsomal Cytochrome P450 1A2 activity (Zhang *et al.* 2019).

3.4.3 Coumarins

The coumarins isofraxidin, asculetin, scopoletin and fraxidin were obtained in more than 80 % of the samples, fraxetin in 50 % while coumarin and psoralen were found in less than 30 % of samples. The concentrations of individual coumarins were in the range between 4.5 and 49.1 ng/L. Isofraxidin, asculetin, scopoletin and fraxidin have been previously detected in water samples from a German floodplain forest at concentrations

up to 157 ng/L (Nanusha et al. 2020b). The same study reported psoralen at lower detection frequency but with concentrations up to 224 ng/L in river waters and thus 45 times greater than the concentration (5 ng/L) in the present study. Coumarins are synthesized by several plants, especially by those of the Apiaceace family (Nakamura *et al.* 2013, Shinbo *et al.* 2006, Whang *et al.* 2005, Lake 1999). Simple coumarins have been found to be biologically active with anti-stress, anti-fatigue, anti-gastric ulcer, anti-depressive, immuno-enhancing and anti-inflammatory effects (Whang et al. 2005, Witaicenis *et al.* 2010). Scopoletin is mainly synthesized by *Scopolia* species, however its presence in river water could also be caused by the massive presence of *Urtica dioica* (Afendi et al. 2012). *In vitro*, scopoletin exhibited acetylcholinesterase inhibition with IC_{50} of 169 µg/L (Hostettmann *et al.* 2006). The exposure to the furanocoumarin psoralen combined with long wave UV radiation causes cytotoxic reactions (e.g. erythema) and genotoxic responses by binding to nucleobases in DNA (Schlatter *et al.* 1991b, Walter *et al.* 1982).

3.4.4 Other miscellaneous compounds

The purine nucleosides, adenine and guanosine were obtained in samples from three rivers at detection frequencies of 80 and 30 %, respectively, and maximum concentrations of about 5 ng/L (Table 1), which are by three orders of magnitude lower than concentrations previously detected in German river waters (Nanusha et al. 2020a). Both compounds are components of all living organisms. Isophorone, synthesized by *Brassica hirta* (Miyazawa & Kawata 2006) and *Prunus armeniaca* L. (Gomez *et al.* 1993), was detected in samples from Kvak Moellebaek and Vejle rivers at up to 25.1 ng/L (Table 1). Its presence in river water originates most likely from human activities, since it is widely used solvent and chemical intermediate. There is no evidence for the presence of plants containing these compounds in the catchments. Chronic (long-term) exposure to isophorone in humans can cause dizziness, fatigue and depression. Animal studies indicate that long-term inhalation of high concentrations of isophorone causes central nervous system effects (USA-ATSDR 2018).

4. Conclusion

This study screened for 160 PSMs in the River Vejle, Denmark, and two tributaries. In total 27 phytochemicals from different compound classes including alkaloids, flavonoids and coumarins were detected in rivers with a minimum of 13 target compounds per sample. Among these PSMs 12 compounds have not been detected in surface waters before. Maximum concentrations of individual compounds reached up to several hundred nanogram per liter. The toxic PAs (intermedine, inchemedine and their N-oxide forms), polyketide-derived alkaloid (coniine) and quinolizidine alkaloids (cytisin and sparteine) were among the detected compounds. The study adds to a series of recent results suggesting that possibly toxic PSMs occur in relevant concentrations in European surface waters and should be considered in monitoring and risk assessment of water resources. Aquatic toxicity data for PSMs are extensively lacking but are required for involving these compounds in the assessment of risks to aquatic organisms and for eliminating risks to human health during drinking water production.

Abbreviations

LCHRMS: Liquid chromatography coupled to high resolution mass spectrometry; PSMs: plant secondary metabolites; KM: Kvak Moellebaek stream; VJ: Vejle river; BS: Ballegab Skovbaek stream; PAs: pyrrolizidine alkaloids; MDL: method detection limit; ND: not detected; LVSPE: large volume solid phase extraction; DI: direct injection.

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Reference

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Supplementary information

for

Occurrence of emerging contaminants of plant origin in river waters from Vejle, Denmark

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Table S1: LVSPE rain event samples collected from Vejle (Haraldskaer); KM - Kvak Moellebaek, BJ - Vejel, BS - Ballegab Skovbaek.

Sample ID	Sampling date	Name of the river
KM_2.10.2019	2.10.2019	Kvak Moellebaek
VJ_2.10.2019	2.10.2019	Vejle
BS_2.10.2019	2.10.2019	Ballegab Skovbaek
KM_4.10.2019	4.10.2019	Kvak Moellebaek
VJ_8.10.2019	8.10.2019	Vejle
BS_9.10.2019	9.10.2019	Ballegab Skovbaek
KM_9.10.2019	9.10.2019	Kvak Moellebaek
VJ_10.10.2019	10.10.2019	Vejle
BS_11.10.2019	11.10.2019	Ballegab Skovbaek
KM_11.10.2019	11.10.2019	Kvak Moellebaek
VJ_11.10.2019	11.10.2019	Vejle
KM_2.11.2019	2.11.2019	Kvak Moellebaek
VJ_3.11.2019	3.11.2019	Vejle
BS_2.11.2019	2.11.2019	Ballegab Skovbaek
KM_16.10.2019	16.10.2019	Kvak Moellebaek
VJ_16.11.2019	16.11.2019	Vejle
BS_16.11.2019	16.11.2019	Ballegab Skovbaek
KM_19.11.2019	19.11.2019	Kvak Moellebaek
VJ_19.11.2019	19.11.2019	Vejle
BS_19.11.2019	19.11.2019	Ballegab Skovbaek

Table S2: Internal standards used for the chemical analysis (ESIpos)

			Used ions (ESI+)		
ID	**Compound name**	**Monoisotopic mass**	**M+**	**M+H+**	**M+NH4+**
IS03	IS03_Mono-isobutylphthalate-D4	226.1143		227.1216	
IS04	IS04_Creatinine-D3	116.0777		117.085	
IS05	IS05_Diazinon-D10	314.1638		315.1711	
IS06	IS06_Benzophenone-3-D5	233.11		234.1173	
IS07	IS07_p-Toluene-sulfonamide-D4	175.0605		176.0678	193.0933
IS10	IS10_1-Naphthol-D7	151.1015			
IS13	IS13_Cotinine-D3	179.1138		180.1211	
IS16	IS16_Bisphenol A D16	244.2155			
IS17	IS17_Diglyme-D6	140.132		141.1392	
IS18	IS18_4-Nitrophenol-D4	143.0521			
IS19	IS19_Chlormequat-D9	131.1296	131.13		
IS22	IS22_Carbamazepine-D10	246.1577		247.165	
IS23	IS23_Triclosan-D3	290.97			
IS24	IS24_Atrazine-13C3	218.1038		219.1111	

IS25	IS25_Estradiol-D3	275.1965			
IS27	IS27_4-Nonylphenol-D4	224.2078			
IS28	IS28_Benzotriazole-D4	123.0735		124.0807	
IS29	IS29_Carbendazim-D4	195.0946		196.1019	
IS30	IS30_Tri-n-butylphosphate-D27	293.3342		294.3414	
IS31	IS31_DEET-D7	198.175		199.1822	
IS37	IS37_Metolachlor-D6	289.1716		290.1788	
IS38	IS38_Isoproturon-D3	209.1607		210.168	
IS39	IS39_Mecoprop-D3	217.0585			
IS40	IS40_Diclofenac-D4	299.0418		300.0491	
IS41	IS41_Caffeine-D3	197.0992		198.1065	
IS42	IS42_Clarithromycin-D3	750.4957		751.503	
IS43	IS43_Desisopropylatrazine-D5	178.0782		179.0855	
IS44	IS44_Decyltrimethylammonium-D30	230.4256	230.43		
IS46	IS46_Laurylsulfate-D25	291.3121			
IS47	IS47_Atenolol-D7	273.207		274.2143	
IS48	IS48_Progesterone-D9	323.2811		324.2883	
IS49	IS49_Verapamil-D6	460.3208		461.3281	
IS50	IS50_Bezafibrate-D4	365.1332		366.1405	
IS51	IS51_Sulfamethoxazole-D4	257.0772		258.0845	
IS54	IS54_Acesulfame-D4	167.019			
IS55	IS55_Tebuconazole-D9	316.2016		317.2089	
IS56	IS56_Hydrochlorothiazide-13C6	302.9846			
IS57	IS57_Imidacloprid-D4	259.0774		260.0847	
IS62	IS62_Bentazone-D6	246.09452			
IS63	IS63_Cyclamate-D11	190.13066			

Table S3: Setting for MZmine data processing.

	Parameters	**Mass detection**	**Chromatogram building**	**Chromatogram deconvolution**	**Join aligner**	**Gap filling**	**Target annotation (identification)**
Peak detection	Mass detector	Centroid					
	Noise level	5.00E+3					
	MS level	1.0					
	Group intensity threshold		1.00E+4				
	Min height intensity		5.00E+03				
	m/z tolerance		0.001				
	Algorithm			Local minimum search			
	Chromatographic threshold (%)			60.0			
	Search minimum in retention time range			0.1			
	Minimum relative height (%)			30			
	Minimum absolute height			5.0E+4			
	Min ratio of peak top/edge			2.3			
	Peak duration range (min)			0.1-0.5			
Peak alignment and identification	m/z tolerance				0.001	0.001	0.001
	Weight for m/z tolerance				70		
	Retention time tolerance (absolute, min)				0.3	0.15	0.5
	Weight for RT				30		
	Intensity tolerance (%)					30.00	
	RT range						
	Adducts						M+H+, M+Na+, M+NH4+,

Table S4: Concentrations (ng/L) of detected targets in individual LVSPE samples from three rivers. (Abbreviations: KM – Kvak Mallebaek, VJ – Vejle river and BS – Ballegab Skovbaek river. Sample code: river_sampling date)

Samples / Compounds	BS_02.10.2019	BS_09.10.2019	BS_11.10.2019	BS_02.11.2019	BS_16.11.2019	BS_19.11.2019	KM_02.10.2019	KM_04.10.2019	KM_09.10.2019	KM_11.10.2019	KM_02.11.2019	KM_16.11.2019	KM_19.11.2019	VJ_02.10.2019	VJ_08.10.2019	VJ_10.10.2019	VJ_11.10.2019	VJ_16.11.2019	VJ_19.11.2019	VJ_03.11.2019
Adenine	ND	ND	0.9	0.5	2.4	1.4	1.9	ND	1.9	1.2	1.6	0.7	1.4	1.6	ND	1.7	2.9	2.9	3.6	6.1
Aesculetin	9.9	10.7	22.6	16.3	10.2	7.0	11.2	11.4	9.4	12.5	16.4	12.3	ND	15.3	13.2	15.5	34.8	13.6	ND	32.0
Coniine	8.9	5.3	7.2	5.9	4.9	3.8	ND	6.3	6.4	6.9	4.9	8.7	3.3	45.7	239.6	108.4	400.5	203.2	68.5	192.2
Coumarin	ND	ND	ND	ND	ND	ND	ND	9.5	5.3	4.5	4.7	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cytisin	6.1	ND	ND	5.3	8.0	4.6	ND	4.8	3.8	5.6	4.5	3.9	8.8	24.8	8.3	ND	9.7	8.4	8.0	7.6
Daidzein	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	88.3	115.2	122.2	84.7	281.9	245.5	148.9
Echimidine	3.4	4.2	4.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Echimidine N-oxide	34.7	29.7	24.7	20.1	15.7	13.5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Fraxetin	13.0	20.3	ND	ND	11.0	ND	13.9	ND	10.3	10.0	16.3	10.2	ND	ND	11.0	ND	29.3	ND	ND	ND
Fraxidin	10.5	13.2	14.7	15.4	12.4	4.9	10.1	15.5	8.2	13.0	16.3	6.9	5.9	7.0	8.4	8.8	9.8	11.1	9.4	13.8
Guanosine	ND	ND	ND	ND	2.4	ND	ND	ND	ND	ND	ND	ND	5.8	ND	2.5	2.8	ND	ND	2.5	2.8
Hordenine	5.5	5.7	8.4	11.0	11.5	7.5	ND	7.2	5.0	11.3	5.6	5.5	9.8	15.5	18.6	13.1	14.7	19.9	21.9	21.4
Hyperoside	11.6	30.9	36.2	20.0	5.5	ND	23.0	28.2	26.6	41.4	42.6	13.5	3.9	37.7	36.1	23.8	51.6	12.1	4.5	3.7
Indole-3-carboxaldehyde	18.2	13.8	17.5	9.3	8.0	5.3	20.1	17.0	7.4	11.8	10.5	7.5	5.9	23.5	19.0	25.8	108.5	17.2	12.2	27.4
Intermedine	4.6	7.1	8.5	3.5	2.2	1.3	3.4	3.9	3.7	ND	2.9	1.2	ND	8.5	7.8	10.7	12.5	4.2	4.5	10.4
Intermedine N-Oxide	24.8	16.5	11.5	16.8	18.1	11.6	4.6	15.8	9.8	10.7	10.3	8.5	4.2	25.5	18.9	27.4	30.0	26.8	23.8	47.0
Isofraxidin	22.9	30.1	21.9	15.7	10.0	5.9	18.3	24.0	16.1	17.8	16.9	6.4	6.3	20.5	26.4	39.6	49.1	17.9	15.2	39.4
Isophorone	ND	ND	ND	ND	ND	ND	25.1	22.1	ND	18.1	20.4	12.2	ND	ND	13.6	ND	ND	ND	ND	ND
Kaempferitrin	8.0	5.5	11.4	19.4	12.6	0.0	5.8	5.8	6.2	0.0	25.1	51.8	26.1	ND	ND	ND	ND	6.8	ND	5.4
Lycopsamine N-oxide	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	3.2	ND	ND	ND	ND	ND	ND	ND
Nicotiflorin	9.0	5.9	26.4	21.5	5.2	ND	15.9	11.2	ND	ND	ND	ND	ND	ND	7.2	ND	6.6	ND	ND	ND
Piperine	ND	ND	ND	ND	ND	ND	ND	ND	18.1	ND	0.5	0.4	1.3	0.3	0.3	ND	ND	ND	0.4	ND
Psoralen	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	5.0
Quercetin	23.5	23.0	ND	ND	ND	ND	23.9	ND	ND	31.2	ND	ND	ND	36.5	33.1	24.9	ND	ND	11.3	ND
Rutin	62.0	28.7	14.6	13.6	ND	5.2	191.0	89.5	30.9	59.7	24.7	7.2	ND	76.3	50.1	24.8	71.3	ND	ND	8.2
Scopoletin	22.6	21.7	17.2	14.6	11.0	7.7	13.4	19.1	14.3	13.1	11.2	6.9	5.5	9.1	11.8	14.0	12.5	10.3	9.9	13.3
Sparteine	6.8	5.3	4.9	4.5	ND	ND	10.8	6.1	5.0	4.8	4.6	4.4	ND	8.4	7.0	9.7	8.2	4.5	ND	6.6