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Occurrence of plant secondary metabolite fingerprints in river waters from Eastern Jutland, Denmark

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Abstract

Background: 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 previous 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.

Results: 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 maximum concentrations of about 400 and 282 ng/L, respectively. In some samples, especially from Vejle river, the rise in concentration of target compounds were associated with increase in the rain intensity and elevated water table in the river.

Conclusions: The measured 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

Background

A myriad of possibly toxic plant secondary metabolites (PSMs) are synthesized by both natural and agricultural vegetation [16, 24]. 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) [32, 77]. The synthesizing plants release these compounds to the environment as leachate, root exudate, and through rain sewers, surface run off and decomposition of plants [3, 4, 20]. According to Günthardt et al. [16], in the environment, many PSMs are mobile which facilitate their transfer to surface

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waters especially during rain events. Hama and Strobel [20] also reported the transfer of alkaloids from plant to soil and pore water as well as their mobility in soil and decline in concentration with depth. Once PSMs enter the environment, they often show similar properties as anthropogenic aquatic pollutants in terms of mobility, persistence and possibly also ecotoxicity [17, 59]. 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 [3, 35, 50, 56, 74, 77]. Due to their high toxicity, PAs might be suspected to contribute substantially to toxic risks if wildlife or humans are exposed via dermal contact or ingestion [15]. 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 [10]. PAs pose genotoxic and carcinogenic risks to animals including humans [50, 74, 76] and induce liver injury in livestock [50]. Flavonoids are widely distributed in a variety of plant species including many edible plants as dietary components [44]. 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 [5] and may inhibit acetylcholinesterase (AChE) [5].

Phytochemicals (toxins) have been studied in food and feed for decades, but little attention has been paid to their occurrence in the environment [8, 11, 24, 33]. Only recently first results on the occurrence of naturally occurring compounds (e.g., PSMs) in water and soil have been reported [17, 19, 21, 25, 48, 49]. Despite the large variety of PSMs that might be released 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. [48] studied the impact of surrounding vegetation on the chemical mixture in river water using LC-HRMS non-target screening (NTS) and identified overlapping chemical signals in plant elutriates and potentially impacted river water. The study revealed thousands of overlapping chemical signals, of which the identities 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 [48]. The study also pointed out the impact of rain intensity on the leaching and run-off of phytochemicals into receiving surface waters. 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 [49]. The occurrence of estrogenic isoflavones (e.g., formononetin and daidzein), indole alkaloids (e.g., gramine) and pyrrolizidine alkaloids (e.g., senecionine and senkirkine) in concentrations of up to 55 ng/L were reported in surface waters from Switzerland [17, 25]. Hama and Strobel [19] 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.

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, 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.

Materials and methods

Water sampling

Our study addressed the Vejle River (Danish: Vejle Å), an approximately 32 km long river, and its two small tributaries (Kvak Moellebaek and Ballegab Skovbaek streams) near to Vejle Municipality in Eastern Jutland, Denmark with one sampling site each (Fig. 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* × uplandicum (comfrey), *Urtica dioica* (common nettle) and grassland with *Senecio Jacobaea* L. (ragwort). In close location to the water sampling sites, all the three rivers share the same vegetation and soil, which is sandy (for more information on soil property see Additional file 1: Table S1).

River water was sampled from October to November 2019, which is a typical rain season. Here, the rain season was selected with the hypothesis that rain washes PSMs from the producing plant or during the decay of wilted plant parts to the receiving river water. As noticed during the sampling campaign, the plant species in the surrounding areas were vital at the beginning of the sampling campaign but turned to decay in November. A total of 20 samples of 20 L of water were extracted on-site at the three sites (see Additional file 1: Table S2) using large volume solid phase extraction (LVSPE) [60] devices from Maxx Mess-und Probenahmetechnik

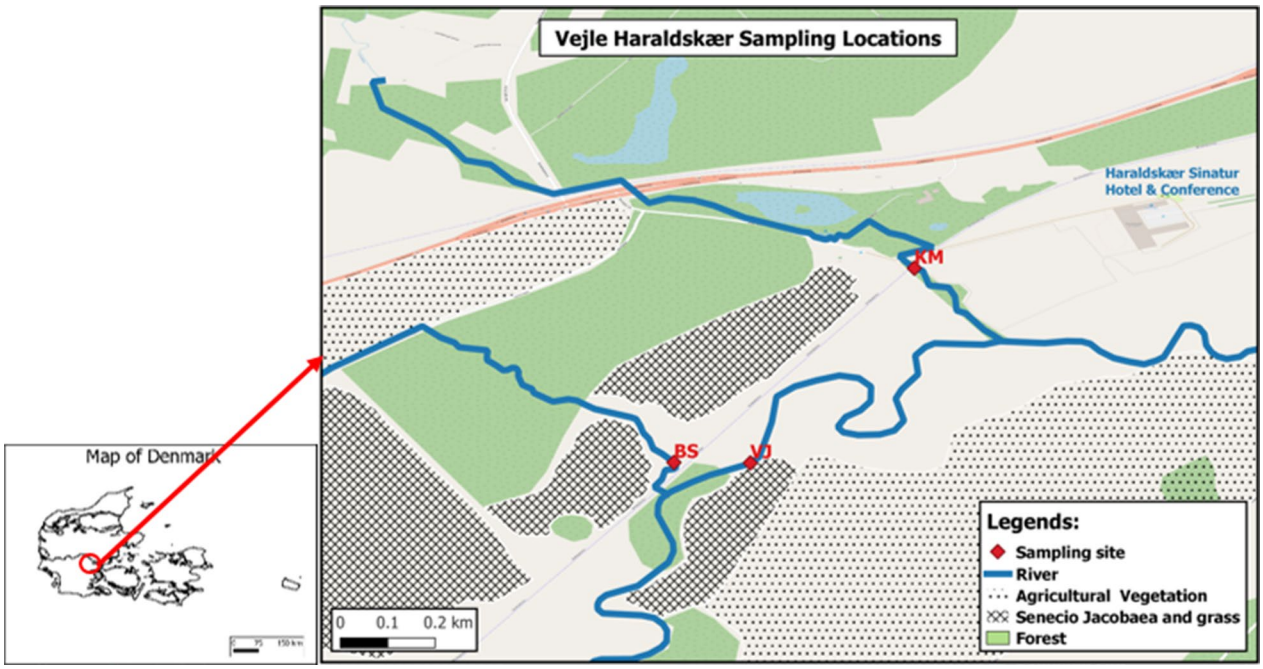

Fig. 1 Map showing water sampling sites. *KM* Kvak Moellebaek stream, *VJ* Vejle river, *BS* Ballegab Skovbaek stream)

GmbH, Rangendingen, Germany triggered by the rise in water level resulting from rain events (Danish Meteorological Institute www.dmi.dk and 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 h with a flow rate of approximately 3 mL/min. During the sampling campaign several rain events occurred (see Additional file 1: Table S3), however, only events that created water table rise in the river/streams for about more than 3 cm were sampled. Extraction cartridges were preconditioned with methanol/ethyl acetate (1:1, v/v), methanol and water [66]. 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.

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 [48, 49] for detailed information).

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 Additional file 1: Table S4) containing isotope-labelled compounds (1 µg/mL), 30 µL of methanol and 60 µL of water. LVSPE using the same solid phase and following the same procedure showed good recovery (50–123%) for more than 80% of 251 organic test compounds covering a broad log D range from −3.6 to 9.4 [60]. Our target compounds with log D of −1.7 to 3.7 (Table 1) were well within that range and thus within the domain of this method.

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)

Table 1 Concentration rage (min–max, ng/L) of individual targets identified in water samples from three rivers

Compound name	Compound class	Formula	CAS no.	*m/z*	RT (min)	log K_{ow}	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	− 0.73	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	0.55	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	2.59	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	1.51	3.6	ND	4.5–9.5	ND
Cytisin	Alkaloid	$C_{11}H_{14}N_2O$	485-35-8	191.1179	0.5	0.60	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	2.55	1.0	ND	ND	84.7–281.9
Echimidine	Alkaloid	$C2_0H_{31}NO_7$	520-68-3	398.217	6.4	2.33	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	1.56	2.7	13.5–34.7	ND	ND
Fraxetin	Coumarin	$C_{10}H_8O_5$	574-84-5	209.0443	6.2	0.38	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	0.42	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	− 1.71	1.5	2.4	5.8	2.5–2.8
Hordenine	Alkaloid	$C_{10}H_{15}NO$	539-15-1	166.1226	0.5	1.53	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	− 0.92	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	1.77	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	0.91	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	0.15	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	0.68	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	2.62	5.5	ND	12.2–25.1	13.6
Kaempferitrin	Flavonoid	$C_{27}H_{30}O_{14}$	482-38-2	579.1707	9.3	− 1.10	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	0.15	0.5	ND	3.2	ND
Nicotiflorin	Flavonoid	$C_{27}H_{30}O_{15}$	17650-84-9	595.165	9.3	− 0.63	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	3.69	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	2.06	2.9	ND	ND	5.0
Quercetin	Flavonoid	$C_{15}H_{10}O_7$	117-39-5	303.0496	8.6	1.48	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	− 1.11	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	0.86	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	2.73	1.7	4.5–6.8	4.4–10.8	4.5–9.7

KM Kvak Moellebaek, *VJ* river, *BS* Ballegab Skovbaek stream, *ND* not detected, *MDL* method detection limit, log K_{ow} evaluated using EPI Suite

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 2 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.

Target screening

Qualitative target screening

For target screening of water samples, the prioritization of target PSMs was mainly built on the previous work by Günthardt et al. [16], identifying plant phytotoxins with a high probability to reach the aqueous environment due to mobility and persistence. Based on in silico predictions, the authors prioritized phytotoxins with half-life longer than 20 days, a log D_{OC} (organic carbon–water partition coefficient) below 4.5, reported rodent or aquatic toxicity and high abundance of the producing plant in Switzerland [16]. Out of these PSMs, we selected about 160 target PSMs (as described by Nanusha et al. [48, 49]) based on their commercial availability and cost as well as the probability of occurrence due to the abundance of the plants identified as the origin of some metabolites. Furthermore, only metabolites containing one or more of the elements nitrogen, oxygen and sulfur, in addition to carbon and hydrogen were selected allowing for electrospray ionization and thus LC-HRMS detection.

The LC-HRMS raw data were converted to mzML format using ProteoWizard (version 2.1.0) [26]. The centroid data were subjected to MZmine (version 2.38) for peak detection followed by peak alignment and target compound annotation [34, 46, 52]. Settings for each step of the data processing are given in Additional file 1: Table S5. 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. 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).

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 [64].

Results and discussion

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 [48, 49] are given in Fig. 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 Additional file 1: Table S6. 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.

In all water samples at least 13 co-occurring targets were detected 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 (Fig. 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 Ballegab Skovbaek.

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 Additional file 1: Table S6. 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 0.3 to 400 ng/L, followed by coumarins (26% of detected targets and a concentrations range of 4.5 to 49 ng/L) and flavonoids (22% of the detected target compounds and concentrations from 3.7 to 282 ng/L).

Dependence of concentrations on rain intensity

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 (Fig. 4a, b). In Fig. 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 [32]. In general, two trends could be observed including rising concentrations with the rise in water level as observed for Vejle river, which is consistent with the leaching hypothesis at heavy rain events (Fig. 4b) and the reduction over time as observed for Ballegab Skovbaek stream, which is consistent with the decline of vegetation in autumn (Fig. 4c). River Kvak Moellebaek exhibits a kind of overlay of both with a general decline over time but a peak concentration at the most severe rain event (Fig. 4a). In samples from Kvak Moellebaek stream (Fig. 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 (Fig. 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

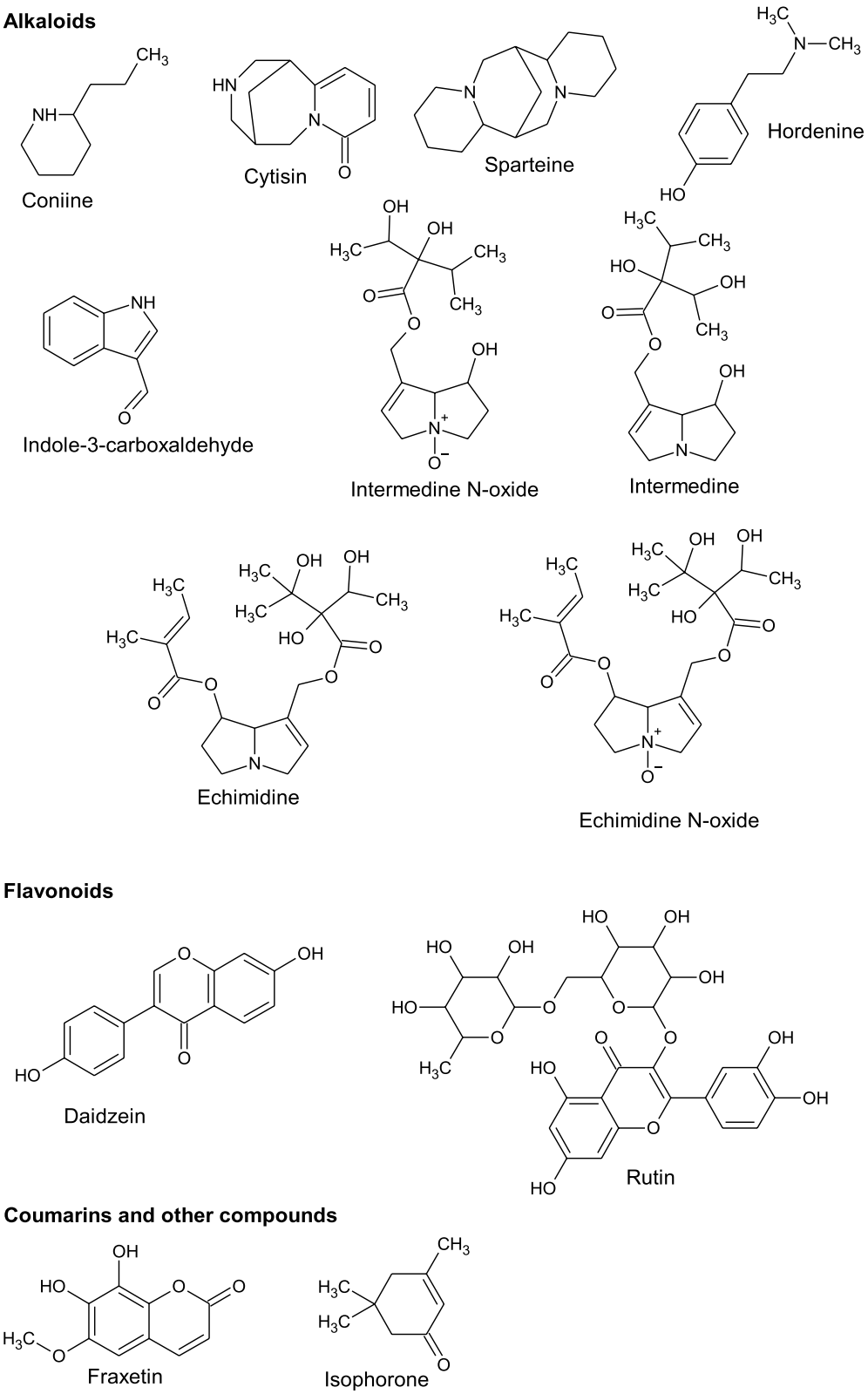

Fig. 2 Chemical structure for some of the detected target compounds (not reported previously by [48, 49])

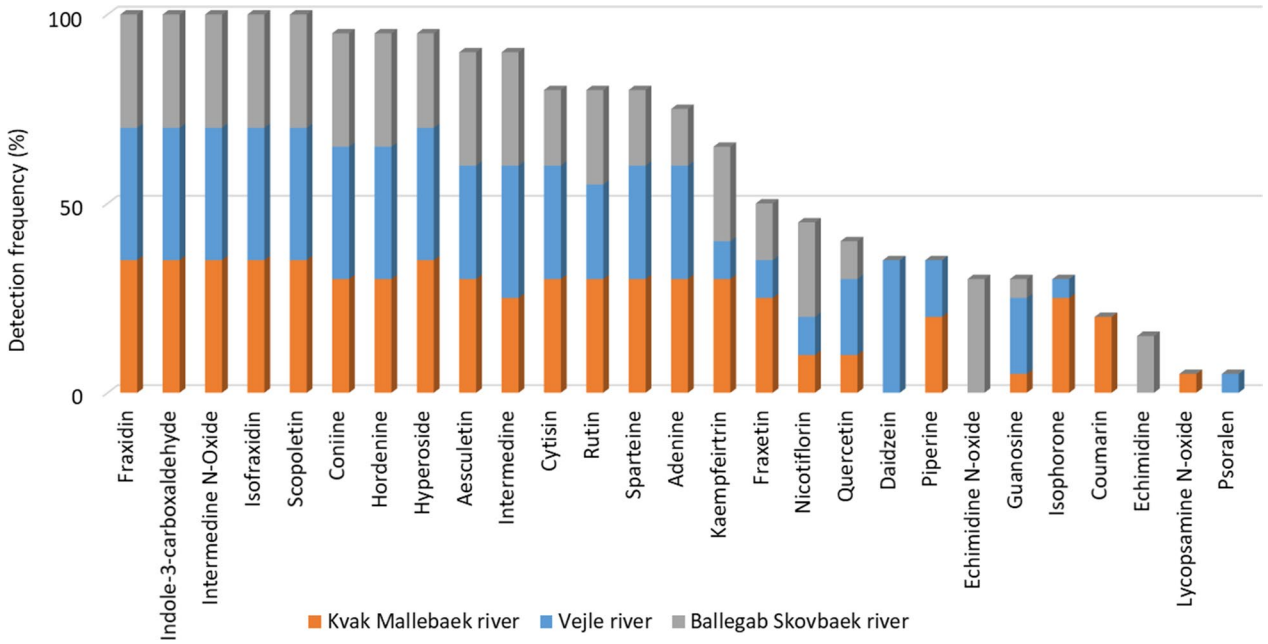

Fig. 3 Detection frequency of the identified target compounds

compound leaching from vegetation during rain. Samples from Ballegab Skovbaek stream (Fig. 4c) showed different behavior of concentrations of target compounds with continuous decline over time, from October to November samples.

Distribution of individual targets

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* [6, 7, 16, 43, 55]. This is in agreement with the abundant presence of *Symphytum* × *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. Similarly hepatic necrosis and ascites were developed by chicken (*Gallus gallus domesticus*) followed by administration of 1.04 mmol/kg body weight per day for 7 days in in vivo study [7]. 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 human and livestock [67]. 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 [19, 21].

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 6, 5.2 and 11.1 ng/L, while the latter reached 5.4, 6 and 7.4 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* [1, 16, 54]. 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 [57, 69].

The phenethylamine alkaloid hordenine was detected in 95% of samples at an average concentration range of 7.4 to 17.9 ng/L (Fig. 3 and Additional file 1: Table S6). 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 [1, 13, 30]. Its detection in the three rivers is in agreement with the abundance of agriculture

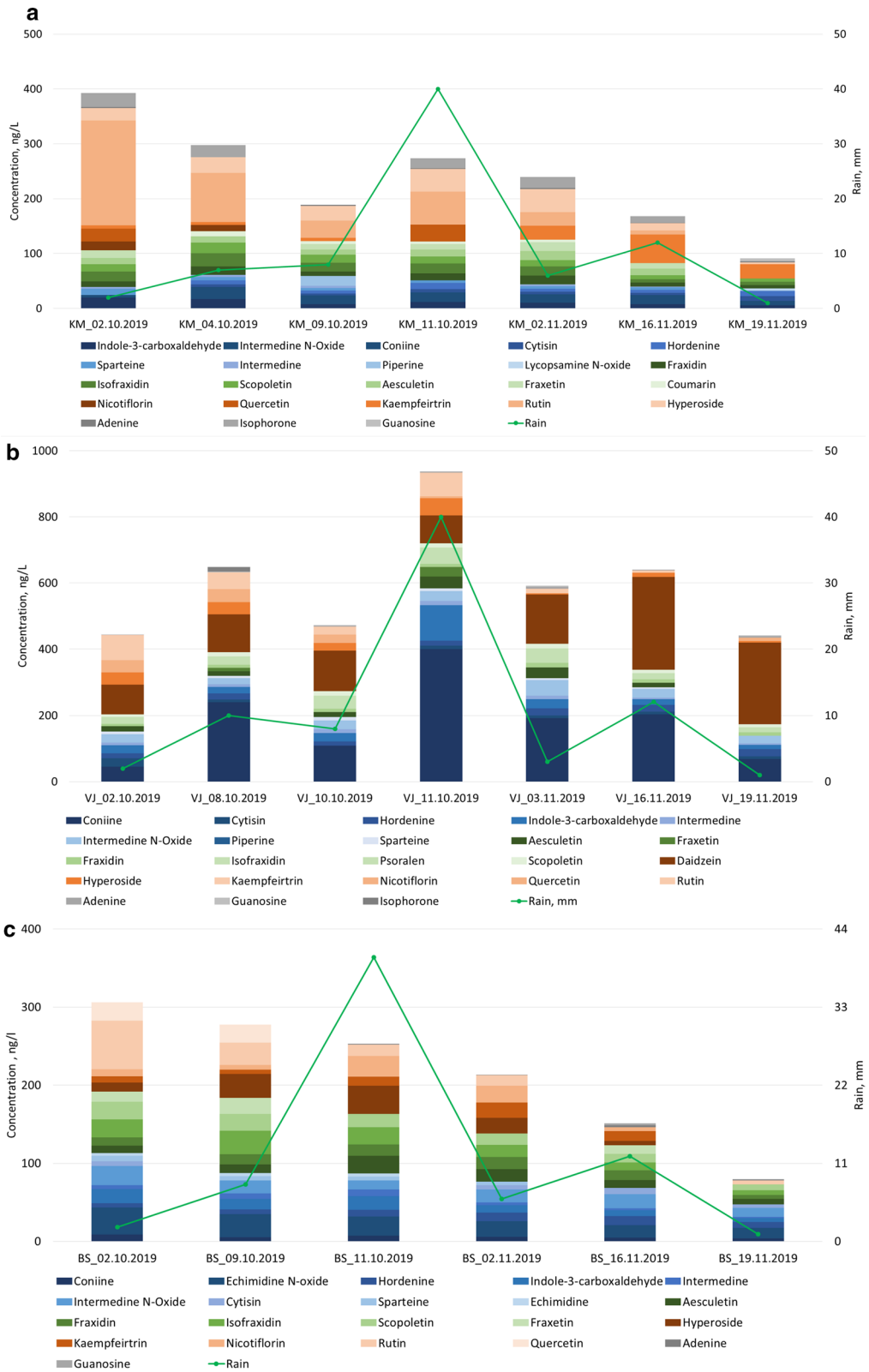

Fig. 4 Sampling date or rain intensity dependent variation in concentration of target compounds identified in water samples per river. **a** *KM* Kvak Moellebaek stream, **b** *VJ* Vejle river and **c** *BS* Ballegab Skovbaek stream, *Sample ID* river name_sampling or rain event date

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 [13, 30, 36]. Hordenine may decrease the UV protection by inhibiting the production of melanin, which plays an important role in protecting skin against ultraviolet light injury [36].

Coniine, a polyketide-derived alkaloid, was detected in 95% of samples from three sites (Fig. 3). It was detected at an average concentration range of 6 to 179.7 ng/L—the highest occurring in samples from Vejle river (Additional file 1: Table S6). Coniine is known to occur in toxic Apiaceae such as *Conium maculatum* [1, 16, 42]. 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 [28, 29, 42]. Coniine's most famous victim is Socrates who was sentenced to death by poison chalice containing poison hemlock in 399 BC [28]. Following the administration of coniine, signs of maternal intoxication were observed in both rat and rabbit [12].

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 [49]. 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 [58, 62]. 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 [9, 51]. Zwart et al. [79] detected piperine in waste water treatment plant effluents and classified it as one of the most potent nonsteroidal estrogens.

Indole-3-carboxaldehyde, an indole alkaloid, was detected in all samples from all three rivers (100% detection frequency—Fig. 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*) [1, 53]

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 [17], up to 40 ng/L in rivers in Iowa [37], while concentrations in the River Danube downstream of untreated wastewater discharge reached almost 500 ng/L [38]. Daidzein is a naturally occurring isoflavonoid phytoestrogen belonging to the non-steroidal estrogens and is mainly derived from the Fabaceae family plants such as soybean, peas and red clover [1, 5, 25, 41]. 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 (resulted from dilution) and supporting the release of daidzein from 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. It has been reported to affect various neurobiological regulatory mechanisms such as behavior, cognition, growth, development and reproduction [2, 40].

Rutin and hyperoside were obtained with a detection frequency of >80% (Fig. 3) and concentrations up to 190.9 ng/L (Table 1). Whereas their aglycone quercetin in 40% of samples at concentration range 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 [1] as well as by *Symphytum officinale* [63]. The detection of rutin at high concentration (190.9 ng/L), among flavonoids, could be linked to the abundant presence of *Symphytum* × *uplandicum*. The occurrence of quercetin and rutin could also be connected with the high abundance of *Urtica dioica* along the rivers [1]. 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 [48]. The authors found quercetin and hyperoside in river waters at considerable concentrations up to 2.5 and 4 µg/L, respectively [48]. Vila-Nova et al. [68] 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. Numerous pharmacological applications were reported for hyperoside, for example for the improvement of the cardiac function and for the

treatment of liver fibrosis [71, 72]. The same compound displayed acetylcholinesterase inhibition and depression of the central nervous system [18, 23].

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 [48]. Nicotiflorin is synthesized by *Urtica dioica* [1]. Both compounds decrease arterial blood pressure and heart beat rate and have hepatoprotective effects [22]. Nicotiflorin was found to protect against memory dysfunction and oxidative stress in multi-infarct dementia model rats [22, 31]. A study by Zhang et al. [78] showed that kaempferitrin competitively inhibited human liver microsomal Cytochrome P450 1A2 activity.

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 [49]. 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 [39, 47, 61, 73]. 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 [73, 75]. Scopoletin is mainly synthesized by *Scopolia* species; however, its presence in river water could also be caused by the massive presence of *Urtica dioica* [1]. In vitro, scopoletin exhibited acetylcholinesterase inhibition with IC_{50} of 169 µg/L [27]. 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 [56, 70].

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 6 ng/L (Table 1), which are by three orders of magnitude lower than concentrations previously detected in German river waters [48]. Both compounds are components of all living organisms. Isophorone, synthesized by *Brassica hirta* [45] and *Prunus armeniaca* L. [14], 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 [65].

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. Detailed spatial and temporal sampling may help to comprehensively explore the seasonal variation of PSMs in aquatic system as well as their fate and distribution in the environment. 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.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-021-00464-4.

Additional file 1. Occurrence of plant secondary metabolite fingerprints in river waters from Eastern Jutland, Denmark.

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Authors' contributions
MYN: conceptualization, sampling, investigation, experimental analysis, data evaluation and visualization, drafted the original version. MK: conceptualization, investigation, writing—review and editing; WB: conceptualization, supervision, writing—review and editing. BGS: conceptualization, editing and reviewing. TS: LVSPE sampling, editing and reviewing. BWS: sampling site selection, sample collection and sampling, editing and reviewing. All authors read and approved the final manuscript.

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Availability of data and materials
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Ethics approval and consent to participate
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Consent for publication
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Competing interests
The authors declare that they have no competing interests.

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