Occurrence of Emerging Contaminants of Plant Origin in River Waters from Vejle, Denmark

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Research

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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 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.

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 highest maximum concentrations.

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.

1. Background

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 and 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 and 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 and 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 and 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 and Strobel 2019; Hoerger et al. 2009b; Günthardt et al. 2020; Hama and 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 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 (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. seneconine 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 seneconine in the concentration range of 4–270 µg/L in surface water impacted by the high abundance of Senecio Jacobaea L. (Hama and 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 (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 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) (Schulze 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 (Macherey-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 LVSP 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 and 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 + NH₄⁺ 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 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 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 (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 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) |
|---------------|----------------|---------|--------|-----|----------|-----------|--------------------------------------|
| Adenine | Purine base | C_{5}H_{5}N_{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_{9}H_{6}O_{4} | 305-01-1 | 179.0336 | 3.6 | 5.0 | 7–22.6, 9.4–16.4, 13.2–34.8 |
| Coniine | Alkaloid | C_{8}H_{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_{9}H_{6}O_{2} | 91-64-5 | 147.0441 | 7.3 | 3.6 | ND, 4.5–9.5, ND |
| Cytisin | Alkaloid | C_{11}H_{14}N_{2}O | 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 | C_{20}H_{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_{8}O_{5} | 574-84-5 | 209.0443 | 6.2 | 3.5 | 11–20.3, 10–16.3, 11–29.3 |
| Fraxidin | Coumarin | C_{11}H_{10}O_{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_{5}O_{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_{9}H_{7}NO | 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 | 316.1252 | 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_{9}H_{14}O_{5} | 78-59-1 | 139.1117 | 9.0 | 5.5 | ND, 12.2–25.1, 13.6 |
| Kaempferitin | 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 |
| Compound name           | Compound class | Formula     | CAS No      | m/z       | RT (min) | MDL, ng/L | Concentration range (min - max, ng/L) |
|-------------------------|----------------|-------------|-------------|-----------|----------|-----------|-------------------------------------|
| Lycopsamine N-oxide     | Alkaloid       | C₁₅H₂₅NO₆  | 95462-15-0  | 316.1751  | 0.5      | ND        | 3.2 – ND                            |
| Nicotiflorin            | Flavonoid      | C₂₇H₃₀O₁₅  | 17650-84-9  | 595.165   | 9.3      | 3.0       | 5.2 – 11.2 – 6.6 – 7.2              |
| Piperine                | Alkaloid       | C₁₁H₁₉NO₃  | 94-62-2     | 286.1434  | 12       | 0.2       | ND – 0.4 – 11.2 – 0.4               |
| Psoralen                | Coumarin       | C₁₁H₆O₃    | 66-97-7     | 187.0388  | 9.2      | 2.9       | ND – ND – 5.0                       |
| Quercetin               | Flavonoid      | C₁₅H₁₀O₇   | 117-39-5    | 303.0496  | 8.6      | 5.3       | 23 – 23.5 – 11.3 – 36.5            |
| Rutin                   | Flavonoid      | C₂₇H₃₀O₁₆  | 153-18-4    | 611.1604  | 8.7      | 5.0       | 5.2 – 7.2 – 8.2 – 76.3             |
| Scopoletin              | Coumarin       | C₁₀H₈O₄    | 92-61-5     | 193.0496  | 7.0      | 1.5       | 7.7 – 5.5 – 9.1 – 14               |
| Sparteine               | Alkaloid       | C₁₅H₂₆N₂   | 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 (Fig. 4a and 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 (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 (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 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.

**Figure 4:** Sampling date or rain intensity dependent variation in concentration of target compounds identified in water samples per river (Abbreviations: (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 ocinale* 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 and Strobel 2020, 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\textsubscript{50} of approximately 26 µg/L and 77 mg/L, respectively, based on in vitro studies (Schmeller et al. 1994; Villalpando-Vargas and 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 (Fig. 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; Houl and 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; Houl and 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 (Fig. 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 and 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 and Rischer 2017). Following the administration of coniine, signs of maternal intoxication were observed in both rat and rabbit (Forsyth and 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 – 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*) (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% (Fig. 3) and concentrations up to 190.9 ng/L (Table 1). Their aglycone quercetin was also detected in 40% of samples at concentrations 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 and Baxter 1999). Nicotiflorin was found to protect against memory dysfunction and oxidative stress in multi-infarct dementia model rats (Huang et al. 2007; Harborne and 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 and 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.

**Declarations**

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**Author’s 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**

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 author's declare that they have no competing interest.

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