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Aquatic occurrence of phytotoxins in small streams triggered by biogeography, vegetation growth stage, and precipitation

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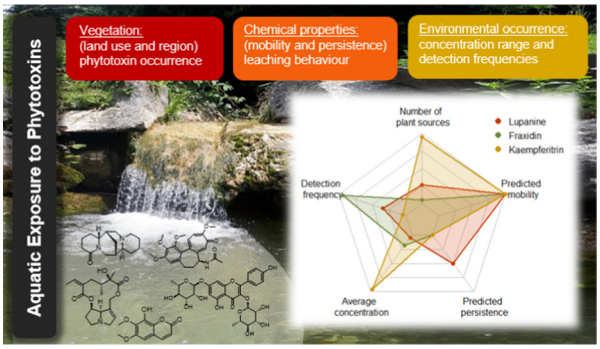
HIGHLIGHTS

- Phytotoxins are toxic plant secondary metabolites with unknown aquatic exposure.
- In total 128 of them were screened in surface water samples and 39 of them confirmed.
- Phytotoxins leach from the biosphere to the hydrosphere.
- Their occurrence was rationalized by vegetation, seasonality and precipitation.
- Phytotoxins' persistence and mobility partly explained aqueous exposure.

GRAPHICAL ABSTRACT



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ABSTRACT

Toxic plant secondary metabolites (PSMs), so-called phytotoxins, occur widely in plant species. Many of these phytotoxins have similar mobility, persistence, and toxicity properties in the environment as anthropogenic micropollutants, which increasingly contaminate surface waters. Although recent case studies have shown the aquatic relevance of phytotoxins, the overall exposure remains unknown. Therefore, we performed a detailed occurrence analysis covering 134 phytotoxins from 27 PSM classes. Water samples from seven small Swiss streams with catchment areas from 1.7 to 23 km^2 and varying land uses were gathered over several months to investigate seasonal impacts. They were complemented with samples from different biogeographical regions to cover variations in vegetation. A broad SPE-LC-HRMS/MS method was applied with limits of detection below 5 ng/L for over 80% of the 134 included phytotoxins. In total, we confirmed 39 phytotoxins belonging to 13 PSM classes, which corresponds to almost 30% of all included phytotoxins. Several alkaloids were regularly detected in the low ng/L-range, with average detection frequencies of 21%. This is consistent with the previously estimated persistence and mobility properties that indicated a high contamination potential. Coumarins were previously predicted to be unstable, however, detection frequencies were around 89%, and maximal concentrations up to 90 ng/L were measured for fraxetin produced by various trees. Overall, rainy weather conditions at full vegetation led to the highest total phytotoxin concentrations, which might potentially be most critical for aquatic organisms.

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1. Introduction

Phytotoxins are toxic plant secondary metabolites (PSMs) with widely varying chemical structures, classified accordingly into alkaloids, terpenoids, steroids, saponins, phenylpropanoids, polyketides, and various smaller classes (Wink, 2003; Wittstock and Gershenzon, 2002). Their toxic modes of action are similarly broad, including allergenic, hallucinogenic, genotoxic, estrogenic or even lethal effects (Poutaraud et al., 2017; Teuscher and Lindequist, 2010). The production of phytotoxins for a plant's protection from any kind of environmental stress is widespread in the plant kingdom, and leads to comparably high PSM concentrations in plants (Poutaraud et al., 2017). Some phytotoxins protect the plants from insects, and were thus already hypothesized as "nature's own pesticides" (Singh et al., 2003). Anthropogenic pesticides are often criticized for their risk posed to surface waters and are thus frequently monitored (Munz et al., 2017; Spycher et al., 2018). Similarly, phytotoxins might be possible aquatic contaminants due to their toxicity and presence in the environment (Bucheli, 2014). However, as they are mostly not considered in monitoring studies, their occurrence remains largely unknown.

Of special concern are persistent, mobile, and toxic (PMT) substances that can reach the aquatic environment and remain there for long periods of time (Reemtsma et al., 2016; Schulze et al., 2019). Based on *in-silico* PMT predictions, we have shown that many phytotoxins are persistent and mobile enough to reach surface waters (Günthardt et al., 2018), and Schönsee et al. (2021) have confirmed the high mobility of many phytotoxins through laboratory experiments. Recently, Nanusha et al. (2021) revealed that a large fraction of unknown substances occurring in surface waters derive from plant species. These unknown plant substances likely include phytotoxins.

Progress on the aquatic occurrence has been made for few individual phytotoxins. For example, aristolochic acids (nephrotoxic and carcinogenic phytotoxins from *Aristolochia clematitis*) were found to contaminate Serbian groundwater in the low ng/L-range (Tung et al., 2020), which might cause serious health risks for humans over long time periods. Ptaquiloside, a carcinogenic norsesquiterpene glucoside from *Pteridium aquilinum,* reached toxicologically relevant concentrations in groundwater after rain events (Clauson-Kaas et al., 2014; Rasmussen et al., 2005). Particularly critical are local high plant abundances, often resulting from human activities as in the case of agricultural areas or invasive plant species. Estrogenic isoflavones were regularly detected in river waters in Switzerland and the USA leaching from grassland with *Trifolium pratense* (Hoerger et al., 2009b; Hoerger et al., 2011) or soybean (*Glycine* max) cultivation (Kolpin et al., 2010; Smalling et al., 2021), respectively, with maximal concentrations over 200 ng/L. Most recently, genotoxic pyrrolizidine alkaloids were regularly found in small creeks with increased concentrations next to plant hot-spots, in some cases due to invasive species (Günthardt et al., 2020; Günthardt et al., 2021; Hama and Strobel, 2019; Hama and Strobel, 2021; Kisielius et al., 2020). Toxic effects of benzoxazinones and the sesquiterpenoid artemisinin to aquatic non-target organisms were found to be similar to those of anthropogenic pesticides (Fritz and Braun, 2006; Jessing et al., 2009). Considering the impact of mixture toxicities, the high diversity of different phytotoxins might add up various adverse biological activities (Altenburger et al., 2015). For example, carboline alkaloids, detected in the Rhine river, were found to exhibit co-mutagenicity with anthropogenic aromatic amines over a synergistic mechanism (Muz et al., 2017). In summary, these examples of the occurrence and toxicity of phytotoxins indicate a possible risk for the aquatic environment.

As such, in this study we conducted a detailed occurrence analysis of phytotoxins in small stream waters including seasonal and regional variations by using a multi-residue analytical method covering 134 phytotoxins from various PSM classes. We validated a broad target and suspect screening method applying solid phase extraction (SPE) followed by a high performance liquid chromatography (HPLC) separation coupled to electrospray ionization (ESI) high-resolution mass spectrometry (HRMS) detection. We tested whether the used *in-silico* prioritization based on a PMT assessment (Günthardt et al., 2018) is capable of predicting actual phytotoxin occurrences. Seasonal and meteorological impacts were assessed through monthly sampling of seven sites situated in the canton of Zurich (Swiss Plateau). The monitoring network was complemented with twelve sites from different biogeographical regions sampled at high vegetation periods. All sites had phytotoxins present in their catchments, either in toxic plants as part of their natural vegetation or agricultural land use, or because of human emission. Finally, the phytotoxin occurrence in small stream waters was evaluated with respect to its ecotoxicological relevance.

2. Methods

2.1. Sites and sampling

To investigate seasonal variations and influences of surrounding vegetation, water samples were taken at seven sites in the canton of Zurich, Switzerland (Fig. 1 and Table S1 in the Supporting Information (SI)). Sampling sites had to fulfill a number of conditions: i) presence of a high diversity of toxic plants based on plant observations, ii) catchment areas of small streams covering different land uses (FOEN, 2020a), and iii) possibility to install and retrieve the water sampling devices described below. Toxic plant species were identified from the Toxic Plants–PhytoToxins (TPPT) database differentiating species producing PMT phytotoxins (Günthardt et al., 2018), and plant observations from 2008 onwards were extracted from Info Flora (2020), resolved for 5×5 km^2 areas. Two of these sites received waste water treatment plant (WWTP) effluents upstream of the sampling spot that potentially contained phytotoxins of anthropogenic origin (sites 2 and 3 in Fig. 1). Samples were gathered monthly at all sites from May until October covering most of the vegetation season in 2019. The exact days were, if possible, set such that a rain event was occurring during the sampling period to capture possible washout effects, which has been shown to be a relevant transport process (Günthardt et al., 2021; Hoerger et al., 2009b). In July, two samples were taken consecutively, of which only one covered a rain event to further study the rain impact. Water-level-proportional samples (approximately 1 L, specified in Table S2) were collected over 48 h using automated water sampling devices described in detail elsewhere (Günthardt et al., 2021; Schönenberger et al., 2020). Briefly, the devices were equipped with a submersed sampling bottle, and the exit air flux, i.e. the sampling rate, was controlled with a precision valve (Göldi Präzisionsmechanik AG (Schlieren, Switzerland)). At constant water-level the sampling rate was time-proportional, but during heavy rain events the sampling period was presumably shorter, and the sampling rate rather flow proportional. In a few cases (8%), the sampling bottles were not entirely full, and thus the bottles had to be filled with grab-sampled water (<250 mL) to reach 1 L. A detailed sampling protocol is included in Table S2.

To investigate regional variations and assess the situation in other parts of Switzerland, additional 1 L grab samples were collected in July 2019 (i.e., at high plant biomass, Table S3 for exact dates) at 13 sites across different biogeographical and climatic regions in Switzerland: Swiss plateau (3, one identical to above), Jura (2), Northern flank of the Alps (1), Eastern Central Alps (2), Western Central Alps (2), and Southern flank of the Alps (3) (Fig. 1 and Table S1). The sampled streams were again selected based on vegetation criteria, i.e. different land uses in the catchment and known occurrence of toxic plant species. Grab samples were taken because of practical limitations. All samples were stored in pre-rinsed aluminum bottles at −20 °C until analysis (frozen within 5 h after sampling). Additionally, the temperature was, with few exceptions, measured before sampling. Precipitation data were received from MeteoSwiss (2020). Overall, a total of 62 water samples were collected for analysis.

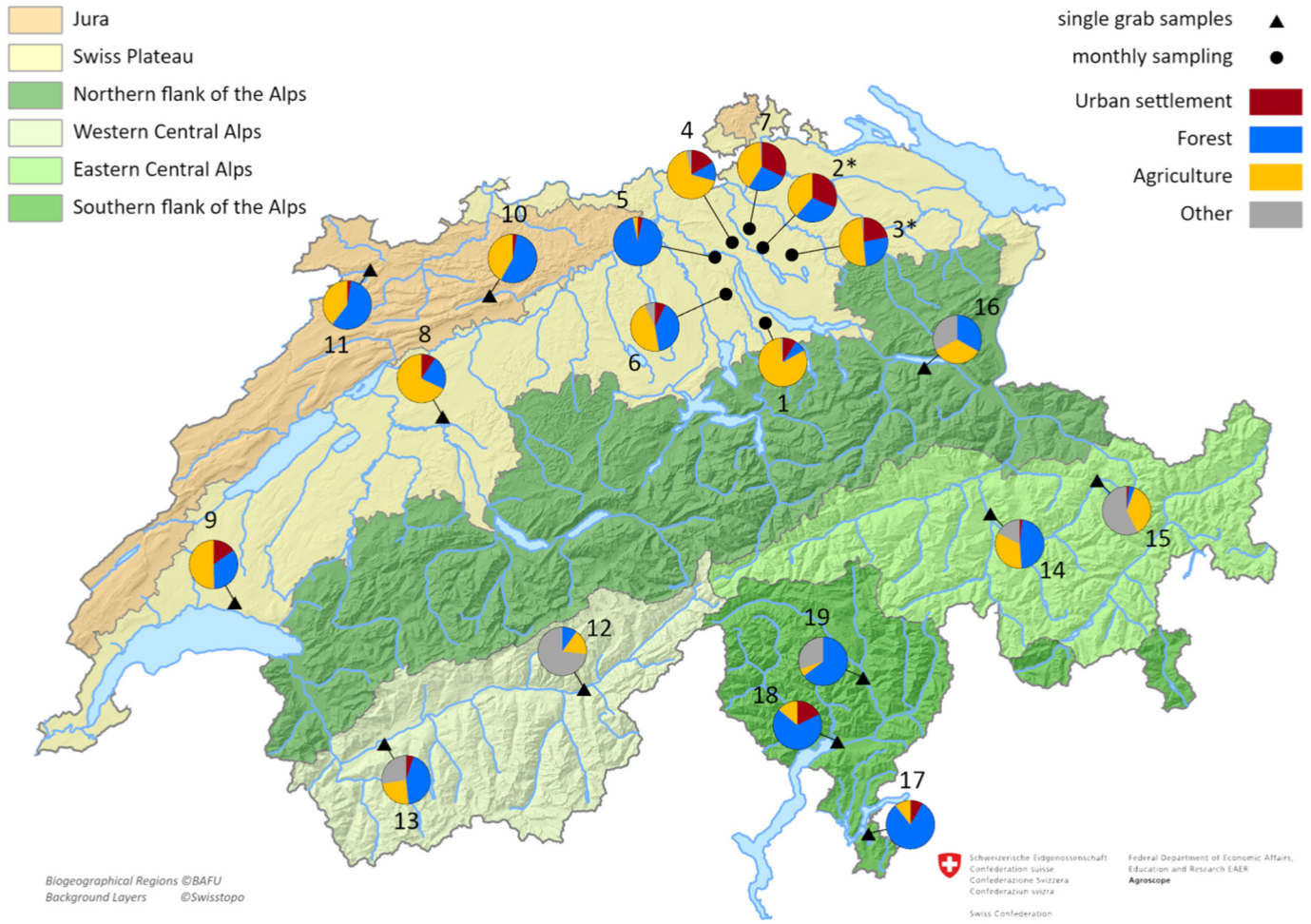

Fig. 1. Map of Switzerland with locations of all small stream sampling sites, monitored either monthly over 48 h from May to October 2019 to study seasonal variations (black dots), or grab sampled once in July/August 2019 (black triangles) to assess biogeographical differences. The pie charts indicate the land use pattern in the catchment areas of individual sites with the gray color representing mostly areas above the timberline. The star indicates sites that receive WWTP effluents. For more details on the sites, see SI (Table S1). Source of land uses of catchments: FOEN, 2020a; Source of biogeographical regions: FOEN, 2020b; source of background layers: Swisstopo, 2020.

2.2. Phytotoxin selection

The phytotoxin selection for this monitoring study was based on our earlier TPPT database compilation aiming for a representative list of targets (Günthardt et al., 2018). Within all phytotoxins, reference standards were selected based on the following criteria: I) PSM class coverage for a broad assessment of the method performance (55% of all targets are classified as PMT), II) expected analytical suitability, e.g. nitrogen-containing phytotoxins accounted for 59% of all reference standards, III) reference standard availability, and IV) priority on phytotoxins with higher occurrence frequencies in plants, i.e. toxins appearing in several plant species or being the major toxin in at least one plant species were chosen. Finally, 134 phytotoxin reference standards were purchased belonging to the following PSM classes: alkaloids including (in alphabetical order) amaryllidaceae alkaloids (3), indole alkaloids (10), isoquinoline alkaloids (9), piperidine alkaloids (2), pyrrolizidine alkaloids (28), quinoline alkaloids (2), quinolizidine alkaloids (5), tropane alkaloids (2), the two isoprenoid alkaloid classes steroidal alkaloids (5) and terpenoid alkaloids (2), and other alkaloids (6); phenylpropanoids including benzoquinones (2), coumarins (8), furanocoumarins (4), naphthoquinones (1), and other phenylpropanoids (2); polyketides including flavonoids (6), isoflavones (2), and other polyketides (1); terpenes including diterpenes (5), monoterpenes (1), saponins (4), sesquiterpenes (9), steroids (5) and triterpenes (3); as well as amides (2), benzoxazinone (1), glucosinolates (1) and one transformation product (1), lignans (1), and naphthalene- and anthracene-derivatives (1). A detailed list with all phytotoxins is given in Table S4.

2.3. Sample preparation

Analytes were solid-phase extracted using a previously developed protocol shown to be successful in pesticide, pharmaceuticals or transformation product screenings (Kern et al., 2009; Munz et al., 2017). The method was initially optimized to cover a broad range of the pH dependent octanol–water partition coefficient (log D_{ow} (pH 7): −4.2 to 4.2) including neutral molecules, but also cations and anions (Huntscha et al., 2012). Over 80% of the phytotoxins are contained in this range based on predicted D_{ow} values from Günthardt et al. (2018). Briefly, 1 L water samples were pH adjusted to 6.5 and filtered (glass fiber filter, 0.7 μm, ∅ 47 mm, Macherey-Nagel (Düren, Germany)). For the following offline SPE, self-prepared multilayer cartridges were used which consisted of three layers: Oasis HLB (200 mg, Waters (Milford, MA)), a mixed-layer with Strata X-AW (100 mg, Phenomenex (Torrance, CA)), Strata X-CV (100 mg, Phemomenex) and Isolute ENV+ (150 mg, Biotage (Uppsala, Sweden), and the final layer of ENVICarb (200 mg, Supelco Sigma Aldrich (St. Louis, MO)). Cartridges were conditioned with 10 mL MeOH and 15 mL water (MilliQ quality), and eluted in the opposite direction with 6 mL of ethylacetate and MeOH (50:50) containing 0.5% ammonia, 3 mL of ethylacetate and MeOH (50:50) containing 1.7% formic acid, and 2 mL of MeOH. The extracts were evaporated to 100 μL using a nitrogen stream and diluted

with water to 1 mL final sample. Details on the chemicals used are given in the SI (S1.2).

2.4. Chemical analysis

Measurements were carried out with HPLC coupled to ESI-HRMS using a QExactive Plus (Thermo Fisher Scientific (Waltham, MA)). For the chromatographic separation, a reversed-phase Atlantis T3 C_{18} column (3 μm, 3.0 mm i.d. × 150 mm, Waters (Milford, MA)) was used with a water-MeOH gradient (both acidified with 0.1% formic acid) and a long gradient over 29.5 min. The ESI interface was operated in negative and positive mode in two separate measurements for each sample. Full scan HRMS spectra were acquired at a resolution of 140′000 (at *m/z* 200) and an *m/z* range from 100 to 1200 or 1000 in positive and negative mode, respectively. The resulting mass error was generally below 5 ppm. Data-dependent HRMS/MS were obtained through higher energy collision-induced dissociation (HCD) triggered over an inclusion list containing all suspects with mass-correlated normalized collision energies (Table S6) at a reduced resolution of 17′500 (at *m/z* 200). Details on the LC gradient and the QExactive settings are given in the SI (S1.3).

2.5. Quantification, method performance, and suspect screening

Quantification was conducted with XCalibur 4.1 (Thermo Fisher Scientific) over an external calibration curve in extracted tap water with 11 standards ranging from 0.25 ng/L to 500 ng/L. Tap water was used as a reproducible and permanently freshly available proxy for the various surface waters because of their matrix variability. Consequences of this approximation are discussed below. Blank tap waters were included over the entire method for quality control and background correction. Full scan measurements were used for the quantification assessing the exact mass integration and retention time (RT) (in Quan Browser), and fragmentation spectra were evaluated manually for confirmation (in Qual Browser). Ionization mode ($[M + H]^+$ or $[M - H]^-$), exact mass-to-charge ratios (*m/z*), RT, and collision energy are listed in Table S6. For the method validation, we determined different parameters in duplicates. Limits of detection (LODs) and limits of quantification (LOQs) were calculated from the signal-to-noise-ratio (lowest calibration standard with detection) using a factor of three and ten for the LOD and LOQ, respectively. For the absolute recoveries, triplicate spiking experiments were carried out in tap water at two different concentrations: 10 ng/L and 200 ng/L. The experiments were performed by adding the standard mixture to tap water at the beginning (before sample filtration) or after the SPE extraction in analogy to the calibration standards. The absolute recovery was then calculated by dividing the area of the prior spiked samples (corrected for possible background contaminations) by the area in the corresponding calibration standard. The method precision was defined as the standard deviation of the recovery calculations, whereas for the instrument precision a sample was measured three times (again for 10 ng/L and 200 ng/L). Please note that matrix effects were not considered and therefore we have to assume an uncertainty in the reported concentrations up to 50% (Huntscha et al., 2012; Günthardt et al., 2021; Hama and Strobel, 2019).

The target quantification was complemented with a suspect screening to expand the target list and cover more phytotoxins. Briefly, an automated suspect hit identification was done in Compound Discoverer 3.1 (Thermo Fisher Scientific) using a suspect list from Günthardt et al. (2018) available over the NORMAN suspect list exchange (NORMAN SusData, 2020). The detected suspect hits were visually filtered according to intensity and peak shape, and evaluated considering RT, isotope pattern, occurrence pattern within the samples, MetFrag prediction, and if available reference spectra. All details on the suspect screening are included in the SI (S1.4 and S2.4).

3. Results and discussion

3.1. Method performance

The SPE-LC-HRMS/MS method was comprehensively evaluated for the quantification of 134 target phytotoxins in a stream water matrix. Table S6 gives the figures of merits for each included phytotoxin. Absolute method recoveries in tap water varied from 30% to 117% at 10 ng/L and from 36% to 124% at 200 ng/L (Figs. 2a and S2). Overall, 91% (10 ng/L) and 87% (200 ng/L) of the included phytotoxins were in the acceptable range of recovery between 70% and 130% (Fig. S3), indicating that no major losses occurred during SPE. Only for goitrin (glucosinolate transformation product) and epigallocatechin (polyketide) recoveries were below 30% and these two substances were therefore not quantified. The instrument precision was on average 3% and varied between 1% and 11% and between 1% and 36% at 10 ng/L and 200 ng/L, respectively. The method precision was on average 12% (10 ng/L) and 13% (200 ng/L), ranging from 3% to 55% (10 ng/L) and 3% to 73% (200 ng/L). The somewhat weaker performance in precisions at high concentrations is explained by those phytotoxins with high LODs (i.e., performing badly) that were excluded at the low, but included in the high concentration data evaluation (i.e. goitrin, diosgenin, or 8-prenylnaringenin). However, in general the LODs were low with a median of 0.4 ng/L and 81% of the phytotoxins having an LOD below 5 ng/L. Correspondingly, the overall median LOQ was also low with 1.4 ng/L. Only for three phytotoxins the LOD was above 100 ng/L, and three phytotoxins were not detectable with this method (aescin, α-humulene, and isopimaric acid). The LODs strongly varied for different PSM classes (Fig. 2). Since the recoveries were largely in the acceptable range, loss of analytes was not assumed as a major driver for increased LODs. Instead, the ionizability of the different PSM classes was more relevant. Most alkaloids and terpenoid alkaloids (>90%) are positively charged at the acidic pH of the mobile phase (pH = 3) and had therefore the lowest LODs (Fig. 2b). In contrast, the values for terpenoids, polyketides, and phenylpropanoids were more variable. These variations can be rationalized by the higher structural differences within these PSM classes and the corresponding impact of the functional groups on the charge transfer potential. Often glycosylated structures had comparably higher LODs. For example, three of four included saponins had an LOD over 30 ng/L and one was not detectable, possibly due to fragmentation of the parent compound. The LODs of the alkaloid terpenoids show a mixed behavior accounting for both structural units (see Fig. 2). Due to the high sensitivity, the calibration curve for 61 phytotoxins (with few exceptions alkaloids) was only linear until 200 ng/L, which was then used as the highest calibration point. Overall, the phytotoxin concentrations reported in the following might be underestimated, because matrix effects were not considered in this work. Huntscha et al. (2012) determined an average ion suppression for polar organic micropollutants in surface water of 34% and in phytotoxin studies matrix effects up to 50% were found (e.g. Günthardt et al., 2021; Hama and Strobel, 2019). The method was more sensitive in positive ionization mode for a majority of the phytotoxins (84%). The negative mode was limited to polyketides, phenylpropanoids, few isoprenoids, and three small PSM classes (glucosinolates, naphthalene- and anthracene derivatives, and benzoxazinones). The alkaloids were with two exceptions not detected in negative mode. The obtained figures of merit characterizing the method performance were similar to those determined by Huntscha et al. (2012) for anthropogenic compounds using the same method. Picardo et al. (2020) developed a similar method based on SPE-LC-HRMS/MS for a partly similar set of natural toxins including also cyanotoxins and mycotoxins, but obtained higher LODs (all natural toxins: 20–1220 ng/L, phytotoxins only: 40–90 ng/L), most probably due to a tenfold smaller enrichment factor.

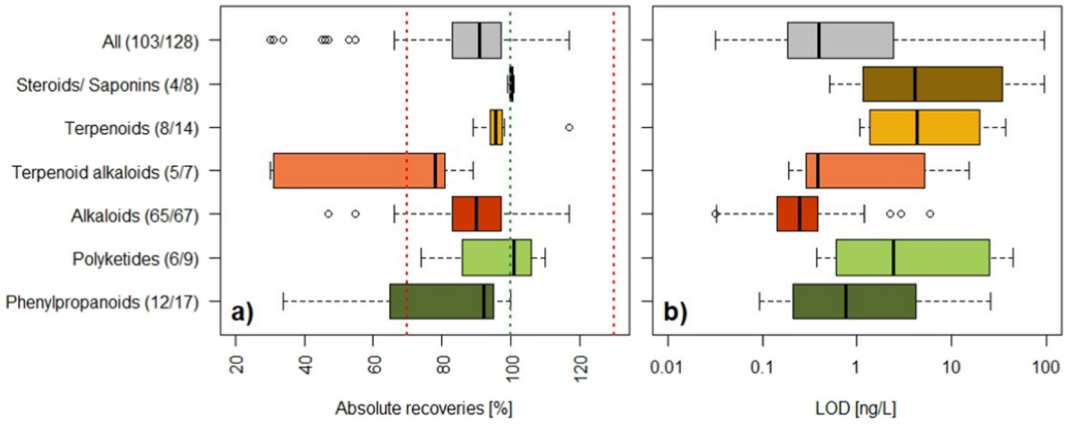

Fig. 2. a) Absolut method recoveries of target phytotoxins at 10 ng/L with the green line indicating the ideal and the red lines indicating the acceptable range (Fig. S2 shows data at 200 ng/L), and **b)** limits of detection (LODs) on a logarithmic scale (only below 100 ng/L included), both determined in tap water and separated into different plant secondary metabolite classes. Numbers in parentheses give the total included phytotoxins per class. Boxplot definitions: line in box is the median (50th percentile), box margins are the 25th and 75th percentile; lines with whiskers are the 10th and 90th percentile, and empty dots are outliers.

3.2. Detected phytotoxins in small streams

We screened all 62 stream water samples from all 19 locations (Fig. 1) for 128 phytotoxins (LOD <100 ng/L) using the known RTs, exact masses, and fragmentation spectra. In total, 39 phytotoxins were confirmed and quantified in at least one sample as summarized in Table 1, which corresponds to almost 30% of the included phytotoxins. Nevertheless, concentrations and phytotoxin patterns strongly varied

Table 1
Detected phytotoxins in stream water samples, including plant secondary metabolite (PSM) class and subclass, maximal and median concentration, samples with detects in % (total 62), and number of sampling sites with detects (total 19 sites).

Phytotoxin name[a]	CAS	PSM class	PSM subclass (abbreviation)	Maximal conc. [ng/L]	Median conc. [ng/L]	Samples with detects [%]	Sites with detects [%]
Hordenine[b]	539-15-1	Amine	–	17	1	58	53
Emodin[b]	518-82-1	Anthracene-derivative	–	11	7	6	11
Vincamine	1617-90-9	Alkaloid	Indole alkaloid (IA)	1	1	5	5
Gramine	87-52-5	Alkaloid	Indole alkaloid (IA)	70	4	50	37
Indole-3-carboxyaldehyde	487-89-8	Alkaloid	Indole alkaloid (IA)	12	4	98	95
Piperine[b,c]	94-62-2	Alkaloid	Piperidine alkaloid	8	2	23	16
Caffeine[c]	58-08-2	Alkaloid	Purine alkaloid	98	10	94	89
Nicotine[c]	65-31-6	Alkaloid	Pyridine alkaloid	2200	21	95	84
Echimidine	520-68-3	Alkaloid	Pyrrolizidine alkaloid (PA)	1	1	5	11
Echimidine N-oxide	41093-89-4	Alkaloid	Pyrrolizidine alkaloid (PA)	4	0.9	19	26
Intermedine	10285-06-0	Alkaloid	Pyrrolizidine alkaloid (PA)	0.5	0.3	5	16
Intermedine N-oxide	95462-14-9	Alkaloid	Pyrrolizidine alkaloid (PA)	2	0.6	10	21
Lycopsamine[b]	10285-07-1	Alkaloid	Pyrrolizidine alkaloid (PA)	0.4	0.2	5	15
Lycopsamine N-oxide	95462-15-0	Alkaloid	Pyrrolizidine alkaloid (PA)	1	1	10	21
Retrorsine	480-54-6	Alkaloid	Pyrrolizidine alkaloid (PA)	1	0.7	15	11
Retrorsine N-oxide	15503-86-3	Alkaloid	Pyrrolizidine alkaloid (PA)	8	3	19	11
Senecionine	130-01-8	Alkaloid	Pyrrolizidine alkaloid (PA)	2	0.6	18	11
Senecionine N-oxide	13268-67-2	Alkaloid	Pyrrolizidine alkaloid (PA)	6	2	29	42
Seneciphylline	480-81-9	Alkaloid	Pyrrolizidine alkaloid (PA)	0.7	0.7	5	15
Seneciphylline N-oxide	38710-26-8	Alkaloid	Pyrrolizidine alkaloid (PA)	9	0.6	32	47
Senecivernine N-oxide	101687-28-9	Alkaloid	Pyrrolizidine alkaloid (PA)	2	1	18	21
Senkirkine	2318-18-5	Alkaloid	Pyrrolizidine alkaloid (PA)	0.9	0.9	10	26
Cytisine	485-35-8	Alkaloid	Quinolizidine alkaloid (QA)	5	0.6	23	37
Lupanine	4356-43-8	Alkaloid	Quinolizidine alkaloid (QA)	4	0.6	34	37
Colchicine	64-86-8	Alkaloid	Tropolone alkaloid	3	2	6	5
Dimethylfraxetin	6035-49-0	Phenylpropanoid	Coumarin (CU)	4	0.5	81	68
Esculetin[b]	305-01-1	Phenylpropanoid	Coumarin (CU)	13	4	98	100
Fraxetin[b]	574-84-5	Phenylpropanoid	Coumarin (CU)	90	17	74	84
Fraxidin	525-21-3	Phenylpropanoid	Coumarin (CU)	43	4	90	89
Scopoletin	92-61-5	Phenylpropanoid	Coumarin (CU)	26	3	98	95
Umbelliferone	93-35-6	Phenylpropanoid	Coumarin (CU)	11	1	92	74
Bergapten/Heraclin	484-20-8	Phenylpropanoid	Furanocoumarin (FC)	0.3	0.3	2	5
Isopimpinellin	482-27-9	Phenylpropanoid	Furanocoumarin (FC)	0.6	0.6	3	11
Matairesinol	580-72-3	Phenylpropanoid	Lignan	25	25	2	5
2-Hydroxycinnamic acid	614-60-8	Phenylpropanoid	–	11	8	13	21
Rutin	153-18-4	Polyketide	Flavonoid (FL)	140	36	5	11
Kaempferitrin	482-38-2	Polyketide	Flavonoid (FL)	83	21	6	11
Daidzein[b]	486-66-8	Polyketide	Isoflavone (IF)	6	0.9	34	42
Formononetin[b]	485-72-3	Polyketide	Isoflavone (IF)	20	3	50	47

[a] Sorted according PSM class with subclasses in alphabetic order.
[b] Results are semi-quantitative only, because phytotoxin did not pass all method validation criteria (see Section 3.1).
[c] Human source.

between individual samples and PSM classes. All sites were exposed to phytotoxins with total concentrations in individual samples varying between 21 and 2390 ng/L (this exceptional high value was dominated by nicotine, see Section 3.3). The median summed phytotoxin concentration was 85 ng/L, and the total number of detected phytotoxins per site differed with a minimal number of six, a maximal number of 23 and a median of 13. Within all detected phytotoxins, the alkaloids represented the PSM class with the highest number of detected compounds accounting for 23 out of the 39 phytotoxins (Table 1). Further important classes were the phenylpropanoids, especially the coumarins, and the polyketides, i.e. flavonoids and isoflavones. The amine hordenine and the anthracene-derivative emodin were the only included phytotoxins from their respective PSM classes; whereas hordenine was detected rather frequently, emodin was detected only occasionally. Although the alkaloids represented the largest class, their frequencies of detection were with few exceptions comparably low. Higher occurrence frequencies were found for the coumarins and, to a lesser extent, the isoflavones. Also, the concentrations and number of exposed sites were mostly higher for the coumarins and isoflavones compared to most alkaloids. The phytotoxin patterns and concentrations observed were impacted by different environmental factors discussed in the following section. The comparison with literature data is included in Section 3.4.

3.3. Evaluation of the PMT assessment to prioritize phytotoxins

The monitoring confirmed the occurrence of various phytotoxins from different PSM classes in the aquatic ecosystem. All phytotoxins were previously prioritized based on their persistence (half-life > 20 days), mobility (log $D_{oc} \leq 4.5$), acute toxicity (either aquatic or rodent), as well as plant abundance to distinguish those causing theoretically a critical exposure in surface waters (Arp et al., 2017; Günthardt et al., 2018). To compare the theoretical PMT categorization and the actual detection of the different PSM classes, the concept of true and false positives and negatives was used as shown in Fig. 3. Roughly 16% of all monitored analytes were previously predicted to occur and of these **true positives** the majority were alkaloids. Almost an equal phytotoxin fraction, namely 15% of all analytes, were **false positives**, which were detected, but categorized incorrectly as non-priority (transient and/or unstable) substances. These **false positives** were phenylpropanoids and, to a lesser extent, alkaloids (Fig. 3). Contradictory results were most likely related to uncertainties in their predicted stability and to potentially underestimated mass flow from input sources. Most half-lives were estimated with BIOWIN from EPI Suite (2017), however, Gouin et al. (2004) and Aronson et al. (2006) suggested that the reactivity may be overestimated and actual degradation half-lives may be higher than BIOWIN estimates. Therefore, certain phytotoxins might be more stable, e.g. the two pyrrolizidine alkaloids intermedine N-oxide and lycopsamine N-oxide were both estimated by BIOWIN to be unstable, but already regularly detected in a previous study (Günthardt et al., 2021). For several phenylpropanoids, the high number of plant sources (Table S4) might lead to a constant emission, by which relatively unstable substances occur regularly due to continuous input. In addition to simple occurrence data, plant density probably constitutes another important criterion for the level of exposure, but is unfortunately rarely available. A large phytotoxin fraction of 42% was predicted to occur, but not detected, representing the group of **false negatives**. Interestingly, not one phytotoxin of the classes steroids, saponins, terpenoid alkaloids, or terpenoids was detected, although they had high fractions with priority phytotoxins (Fig. 3). Possibly these classes have indeed a lower aquatic occurrence due to various reasons: i) their persistence is lower than estimated, ii) the respective plant species have lower abundances, iii) no leaching or wash-out through rain was taking place. Alternatively, the chosen strategy could have been responsible for the false negatives, because either the analytical method was not sensitive enough for many phytotoxins of these classes, or the monitoring strategy was too general (e.g. in terms of frequency, or site selection). Nevertheless, it seems reasonable to conclude that these classes have a much lower relevance for aquatic exposure in Switzerland. Finally, 30% of the phytotoxins were **true negatives**, i.e. not of priority and concordantly not detected.

3.4. Linking phytotoxin occurrences to vegetation

The phytotoxin patterns in the stream samples are determined by the presence of toxic plant species growing in the catchments, and the presence of these species in turn is influenced by the biogeographical region and land use. The phytotoxins' average concentrations are shown for each site in Fig. 4. On the one hand, there are large, but conceivable differences for the various sites such as the low pattern overlap between the alpine sites (sites 12–15) and the Zurich sites receiving WWTP effluents (sites 2 and 3). One the other hand, Fig. 4 shows that certain phytotoxins have very similar patterns, e.g. the two isoflavones formononetin and daidzein produced both by red clover are grouped together.

So far, detailed and dedicated surface water monitoring studies with plausible source allocation had mainly been conducted for the pyrrolizidine alkaloids (Günthardt et al., 2020; Günthardt et al., 2021; Hama and Strobel, 2019; Hama and Strobel, 2021; Kisielius et al., 2020) and the isoflavones (Hoerger et al., 2009a; Hoerger et al., 2011; Kolpin et al., 2010; Smalling et al., 2021). In this respect, these compounds serve as positive controls for our approach. Summed pyrrolizidine alkaloid concentrations ranged between 0.6 ng/L and 22 ng/L, which is in agreement with our previous results (Günthardt et al., 2020; Günthardt et al., 2021). Most dominant were the pyrrolizidine alkaloids produced by different *Senecio* spp. (cyclic structures, e.g. seneciphylline N-oxide) followed by those from *Echium vulgare* (diester structures, e.g. echimidine N-oxide), with both plant genera having a wide distribution in Switzerland. For the third detected pyrrolizidine alkaloid type (monoester structure, e.g. intermedine N-oxide) so far no distinct plant source could be determined. Making use of the detailed plant occurrences at the sampled sites, we revealed that only *Eupatorium cannabinum* was present at all four sites (3, 10, 11 and 18 in Fig. 1) and, therefore, is the most probable plant source of monoester pyrrolizidine alkaloids. *Eupatorium cannabinum* grows mainly in forest areas that are important in the catchments of all contaminated sites (Fig. 1). Alternative pyrrolizidine alkaloid producers were *Lithospermum officinale* present at three sites or *Borago officinalis* present at two sites. The measured isoflavone concentrations ranged between 0.9 ng/L and 6 ng/l, and between 1 ng/L and 20 ng/L for daidzein and formononetin, respectively. Estrogenic isoflavones are produced by red clover (*Trifolium pratense*), which is a common pasture plant growing throughout Switzerland. Hoerger et al. (2009b) found median surface water concentrations of formononetin of around 5 ng/L and lower concentrations for daidzein, which are similar to our detected concentrations.

As discussed, plant source allocation was possible for these two classes in combination with additional literature on aquatic exposure assessments. For many other classes, the precise plant sources were not identifiable because the phytotoxins are produced by too many plant species. For example, the flavonoid kaempferitrin is produced by at least 24 plant genera (Table S4 gives possible sources), and the low detection frequency was possibly due to its predicted instability (Table S4). At the same time, for certain alkaloids as well as coumarins, a rather clear source assignment was facilitated through the low number of possible plant sources. For three out of four additionally detected alkaloid classes a tentative source was found: the two quinolizidine alkaloids cytisine and lupanine probably originated from *Genista* or *Laburnum* spp., the tropolone alkaloid colchicine from *Colchicum autumnale*, and the indole alkaloid vincamine from *Vinca* spp. In all these cases, both the phytotoxins and their producing plant were present at the same sites. Quinolizidine alkaloids were previously detected in soil pore water from an agricultural lupin field (Hama and Strobel,

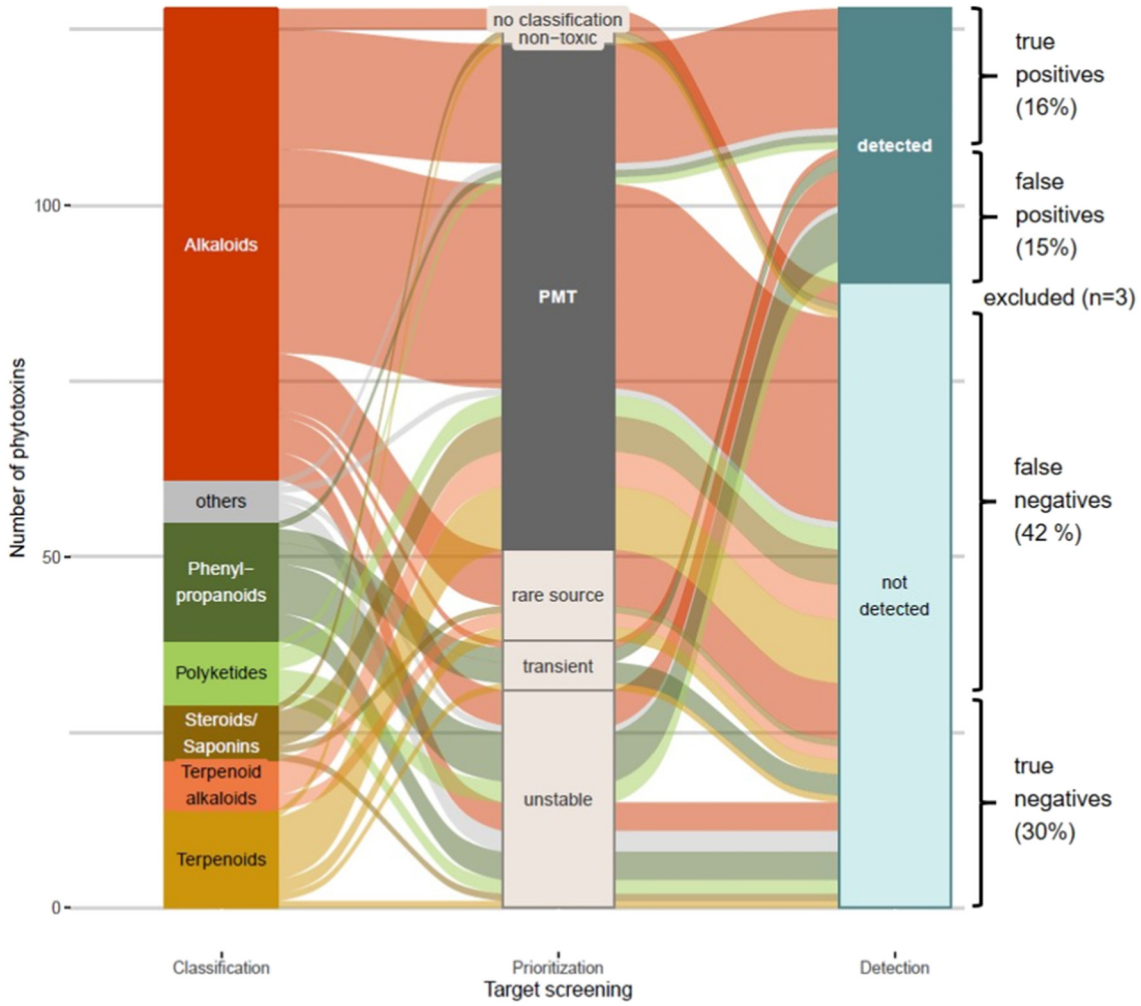

Fig. 3. Distribution of all target phytotoxins ($n = 128$) monitored in small streams among the different plant secondary metabolite (PSM) classes (left column), their *in-silico* predicted persistence, mobility, and toxicity (PMT) assessment (described in detail in Günthardt et al. (2018) and given for each analyte in Table S4; middle column), and the results of the monitoring reported here, i.e. detected or not (right column). The connecting curves show the categorization of phytotoxins of the same PSM class, prioritization, and detection distinguishing **true positives**, **false positives**, **false negatives** and **true negatives**. For example, the broadest curve (in red) indicates the high fraction of alkaloids that were categorized as PMT substances, but were not detected in the monitoring.

2020), which confirmed its mobility. However, its wide distribution in stream waters (10 out of 18 sites contained at least one quinolizidine alkaloid) was not yet known. Two other indole alkaloids, gramine and indole-3-carboxyaldehyde, had various possible plant sources including the agricultural crop *Hordeum*, which resulted in higher concentrations. The structurally similar coumarins showed the highest detection frequencies, and their concentrations were high, e.g. up to 90 ng/L for fraxetin (Table 1 and Fig. 4). For some coumarins, Nanusha et al. (2020a, 2020b) found similar results. Overall, two different characteristics can explain these occurrences. On the one hand, scopoletin and umbelliferone are two of the toxins with the most plant sources, e.g. umbelliferone is produced by over 50 plant genera. On the other hand, fraxetin, fraxidin, dimethylfraxetin, and esculetin are produced by trees, essentially the widely distributed maple (*Acer*), chestnut (*Aesculus*), and ash (*Fraxinus*) trees. The available biomass from trees is clearly higher, which might explain the relatively high concentrations and detection frequencies.

In a comparison of the different sites, a few tendencies became visible. As expected, for the sites monitored monthly, higher toxin numbers were detected (sites 1–7 in Fig. 4). Sites from the same region often clustered together, as shown in Fig. 4, with similar patterns. Overall, the data showed lower phytotoxin numbers for the Alpine site (sites 12–15 in Fig. 4). However, within one biogeographical region also large variations were detected, e.g. at site 17 (Mara, TI, southern flank of the Alps) only 6 phytotoxins were detected, whereas at the site 18 in the same region and sampled on the same day 19 different phytotoxins were detected. These variations might be due to stream size or the impact of the surrounding vegetation, although, no general influence on detected phytotoxins patterns or concentrations was found for the land use patterns. The two sites with the lowest toxin numbers (5 and 6 in Fig. 1) both had high forest fractions. Trees might indeed shield other plants from rain washout, but based on this study it remains uncertain whether catchments with high forest fractions indeed have lower phytotoxin occurrence.

3.5. Phytotoxins of human sources

Three known plant-based, but largely anthropogenic emerging contaminants in aquatic surface waters were included to compare human and natural input sources: nicotine from tobacco, caffeine from coffee consumption, and piperine from pepper used in food flavouring. Table 1 and Fig. 4 clearly show the wide aquatic occurrence of nicotine and caffeine. Nicotine was found with the overall highest maximal concentration of 2200 ng/L, but average concentrations were clearly lower with 21 ng/L, which is largely in agreement with previous studies (Nanusha et al., 2020a; Robles-Molina et al., 2014). The most

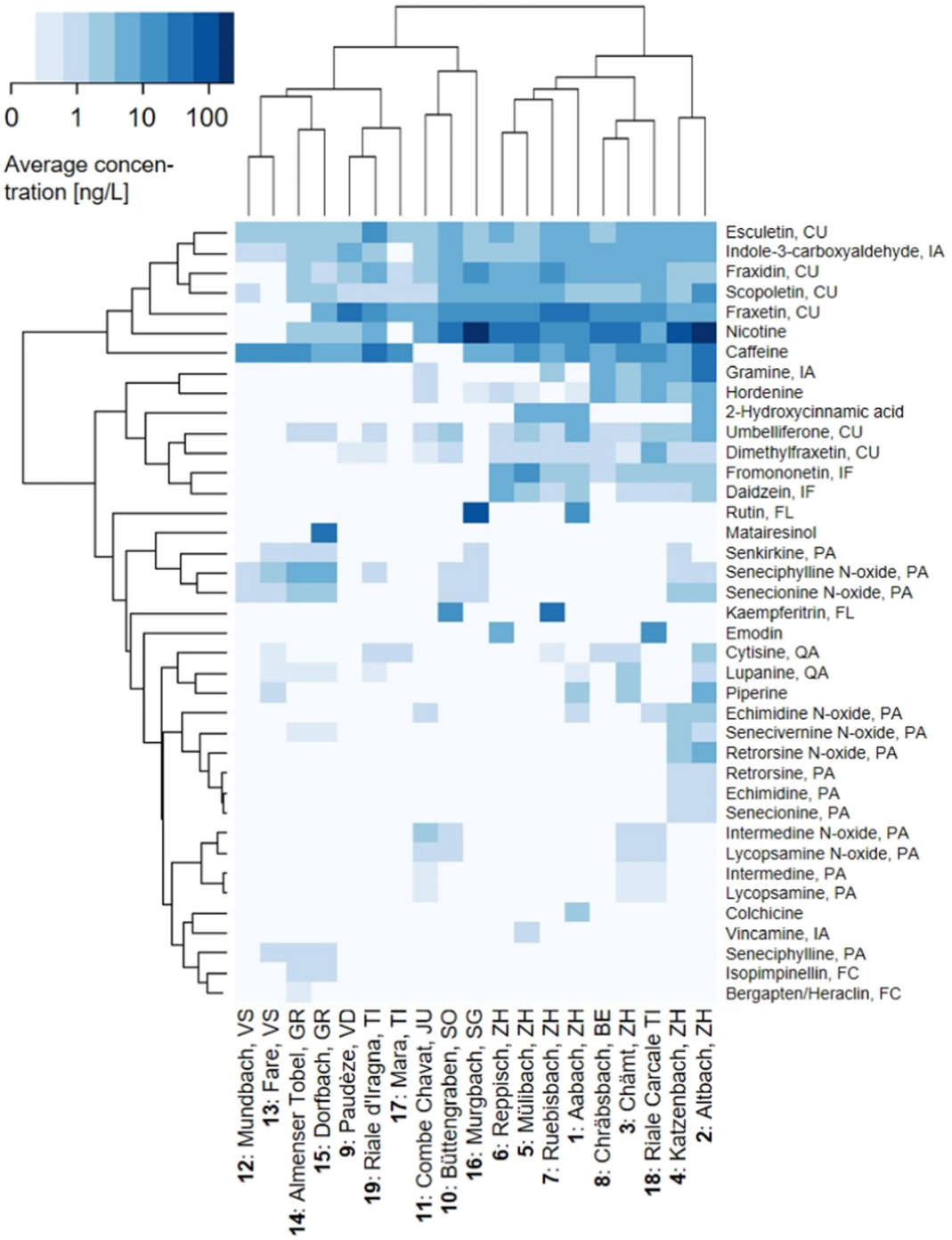

Fig. 4. Phytotoxin patterns for all investigated surface water monitoring sites based on the logarithmic average concentration of each phytotoxin confirmed at least once. The site label includes number, name and canton (for locations, see Fig. 1), the phytotoxin label includes name and PSM class, if more than one phytotoxin of a class was found (coumarin (CU), furanocoumarin (FC), flavonoid (FL), indole alkaloid (IA), isoflavone (IF), quinolizidine alkaloid (QA)).

contaminated site (site 2 in Fig. 1 and Table S1) received WWTP effluents resulting in average concentrations of 365 ng/L, which confirms the high relevance of this contamination source (Buerge et al., 2008). However, another site receiving treated waste water (site 3) had lower average nicotine concentrations (42 ng/L). Other sites without WWTP effluents had similar concentration levels (between 30 and 64 ng/L), which likely derives from tobacco waste, e.g. cigarette residues (Selmar et al., 2018). The alpine sites (12–15 in Fig. 1) had lower nicotine concentrations with no or trace concentrations (below the LOQ), probably because the population density in these areas is lower. Caffeine was less concentrated than nicotine also in comparison with previous studies (Robles-Molina et al., 2014), but was detected more frequently. Similar to nicotine, site 2 showed the highest average concentrations (59 ng/L), whereas most other sites showed levels in the low ng/L-range. Even lower concentrations and detection frequencies were found for piperine, which seems to be less relevant, but similar to different other alkaloids. To conclude, human sources can clearly contribute to the aquatic exposure to phytotoxins, if phytotoxin consumption, excretion and WWTP emission take place. Differentiation between human and natural sources remains challenging, though.

3.6. Linking phytotoxin concentrations with vegetative growth stage and weather conditions

The measured concentrations in the stream water monitoring of phytotoxins were assessed in relation to two important driving factors. First, the influence of the vegetation's growth stage was evaluated through the temporal sampling campaign running between May and October. Secondly, two consecutive samplings in July made it possible to assess the influence of hydrodynamics driven by precipitation on phytotoxin occurrence. The three phytotoxins of human origin are shown in the SI (Fig. S4) and not included in the following since other drivers are responsible for their presence. The development of the total as well as PSM-class-specific concentrations over the growing season are exemplarily visualized for two sites in Fig. 5 (all sites are included in Fig. S4). The summed phytotoxin concentrations (all originating from terrestrial plants) were often highest in summer, where for five out of seven sites the maximal concentrations were found between July and August. In only two sites the maximal concentrations occurred in spring. The lowest concentrations were mostly detected in September and October. Similar patterns with high concentrations in summer were previously found for isoflavones (Hoerger et al., 2009b; Smalling et al., 2021) or pyrrolizidine alkaloids (Günthardt et al., 2021), and explained by the increased biomass of clearly defined species. Despite these general trends, individual phytotoxins and PSM classes did not all show the same temporal patterns. Whereas Fig. 5a shows an example with various classes contributing to the total concentration over time, the total concentration in Fig. 5b is strongly dominated by the coumarins, which accounted for at least 62% and up to 94%. Often, the coumarins show a less distinct temporal pattern. For example, in Fig. 5a the coumarins have relatively constant concentrations around 34 ng/L, while pyrrolizidine alkaloids range from 1 ng/L to 22 ng/L. In Fig. 5b the concentration increases in July and August are mainly due to the addition of the isoflavones and emodin. Umbelliferone and scopoletin are produced by various plant genera (Table S4) and, therefore, the measured pattern is probably an overlap of various sources. For the higher concentrated fraxetin, fraxidin and, to a lesser extent, esculetin and dimethylfraxetin (Table 1), no explanation was found for the rather constant and relatively high concentrations. It is probable that not yet investigated emission processes from trees lead to higher prevalence of coumarins. For example, the shedding of leaves might increase phytotoxin concentrations, such as known for the dissolved organic carbon content (Hongve, 1999; Klimaszyk et al., 2015).

Besides the impact of the season and correspondingly the biomass present, precipitation was identified as an important driver for phytotoxin occurrence, as visualized in Fig. 5 by the two time points in July. Overall, at four out of seven sites (Fig. S4) total concentrations of phytotoxins clearly dropped under dry conditions (by a factor varying between 1.7 and 2.8). For the individual phytotoxins and sites, only in 25% of the cases higher concentrations were found during the dry sampling period, whereas in 67% of the cases concentrations were higher during wet conditions, and no rain impact was detected in 8% (Fig. S5a). The rain impact varied strongly between a negligible amount up to two orders of magnitude and had a peak around a concentration duplication (histogram in Fig. S5b). Therefore, precipitation could be identified as an important mobilization and transport mechanism for aquatic occurrence through rain-induced washout from the plants. The absolute concentration change was probably influenced by a combination of various factors, such as the strength and duration of the rain event, as well as a counteracting dilution effect. Besides that, also other transport processes such as alluvial water exchange might impact the measured concentrations. Similar rain-driven concentration changes were found in earlier dedicated phytotoxin case studies, e.g. for pyrrolizidine alkaloids (Günthardt et al., 2021) or isoflavones (Hoerger et al., 2009b; Kolpin et al., 2010; Smalling et al., 2021), as well as in detailed pesticide monitoring (Strempel et al., 2012).

3.7. Suspect screening

To expand the phytotoxin set beyond the chosen targets, a suspect screening was conducted taking advantage of the HRMS detection and covering over 400 additional phytotoxins (for details see SI Section S2.4). Two additional isoflavones, biochanin A and genistein, were tentatively identified (SI Table S7), which had previously been confirmed and investigated in detail with regard to their aquatic exposure (Günthardt et al., 2020; Hoerger et al., 2009b; Kolpin et al., 2010). Also, the quinolizidine alkaloid hydroxylupanine was tentatively identified, which co-occurred with lupanine. Such a coincidence makes sense: both compounds are produced and emitted by lupines, as recently observed in an agricultural field experiment (Hama and Strobel, 2020). Additionally, the presence of two more fraxidin isomers is likely, because two co-occurring signals were found with similar retention times and fragmentation spectra (Figs. S6 and S7). One is potentially isofraxidin, a coumarin that is produced by the same plant species as fraxidin (He et al., 2009). Although these phytotoxins comprise likely present suspect hits, the number of possible suspects was not very high compared to the much higher number of detected targets. A main reason for this might be that many **false negatives**,

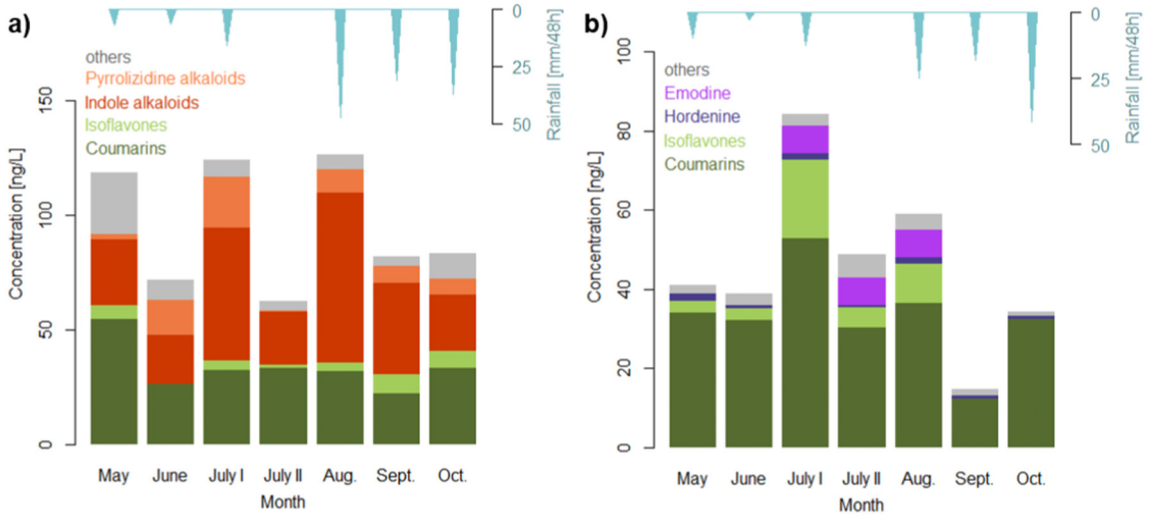

Fig. 5. Total phytotoxin concentrations divided into the main contributing plant secondary metabolite classes or individual compounds detected in small streams between May and October, shown for the two most contrasting sites according to Fig. 4: **a)** site 2: Altbach, ZH and **b)** site 6: Reppisch, ZH (for site information see Fig. 1, for all other sites Fig. S4). Source of rain data: MeteoSwiss, 2020.

i.e. predicted as mobile and persistent, occur below their analytical method detection limits. For example, the tropolone alkaloid vincamine, which constitutes the main toxin in the plant tissue of *Vinca* spp., was detected only in the low ng/L-range. Toxins occurring at low concentrations in plants are expected to have even lower concentrations in surface waters, and their MS/MS were probably mostly not triggered or not of good enough quality to enable a suspect identification. Additionally, phytotoxin structures are often very similar and a structure assignment is not simple, as for the fraxidin isomers. Often, the lack of reference standards also hinders further identification. Nevertheless, the suspect screening confirmed the co-occurrence of additional phytotoxins for different PSM classes indicating the presence of even more complex phytotoxins patterns in stream waters.

3.8. Ecotoxicological relevance

For a proper environmental risk assessment, measured concentrations must be assessed in relation to concentrations that present exotoxicological risks. For the known human-derived emerging contaminants of plant origin, a possible chronic risk was previously found for caffeine and nicotine due to their wide occurrence in surface waters and regular exceedance of no-effect concentrations (for various species) (Oropesa et al., 2017; Rodríguez-Gil et al., 2018). For piperine, effect concentrations were not reached in any of the stream water samples, although it was found to be a potent nonsteroidal estrogen (Zwart et al., 2018). Unfortunately, no ecotoxicological data are available for most other phytotoxins, and a possible risk for aquatic organisms can therefore only be estimated. Based on *in-silico* predictions from ecological structure−activity relationships (ECOSAR, 2017), no acute risk arises from the measured concentrations reported here. For a possible chronic effect, the coumarins might be most critical, because they have the highest concentrations and very high occurrence frequencies. Furthermore, phytotoxins with specific modes of action such as endocrine disruption or genotoxic substances should be considered in-depth. Whereas for the estrogenic isoflavones a possible aquatic effect was largely excluded (Hoerger et al., 2009b), the genotoxic pyrrolizidine alkaloids might pose a risk (Günthardt et al., 2021). Finally, the effect of mixture toxicities always has to be considered because of the co-occurrence of several phytotoxins and in combination with anthropogenic compounds.

CRediT authorship contribution statement

Barbara F. Günthardt: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. **Juliane Hollender:** Conceptualization, Resources, Supervision, Writing – review & editing. **Martin Scheringer:** Conceptualization, Writing – review & editing. **Konrad Hungerbühler:** Conceptualization. **Mulatu Y. Nanusha:** Resources. **Werner Brack:** Resources, Supervision. **Thomas D. Bucheli:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Details on sampling sites and protocols; chemicals, standards and calibrations; instrumental analysis; suspect screening including RT correlation and Compound Discoverer parameters; method performance, all time profiles, and rain impact assessment. Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.149128.

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