Environmental Sciences Europe

RESEARCH Open Access

Toxicity and risk of plant-produced alkaloids to *Daphnia magna*

Megan R. Griffiths, Bjarne W. Strobel, Jawameer R. Hama and Nina Cedergreen*

Abstract

Background: Many plants contain phytotoxic alkaloids to deter herbivorous pests and grazing animals. Alkaloids include quinolizidine and indole alkaloids found in the lupin (*Lupinus* spp.), an ornamental flower and emerging protein crop, as well as pyrrolizidine alkaloids in the ragwort (*Senecio jacobaea*), an invasive, weed-like flower. When lupins and ragworts are present in large densities in fields, there is a concern that alkaloids may leach into freshwater environments in amounts that may affect non-target organisms, such as *Daphnia magna*. This study aimed to investigate (i) the acute toxicity of alkaloids (gramine, heliotrine, lupanine, lupinine, monocrotaline, monocrotaline N-oxide, senecionine and sparteine) in *D. magna*, (ii) the contribution of these individual alkaloids to lupin plant extract toxicity, (iii) the longer term reproductive effects of a representative alkaloid, sparteine, and conclude with (iv) a tentative risk assessment for the sum of alkaloids measured in soil and surface waters.

Results: The alkaloids exhibited toxicity, with 48 h EC_{50} values in the range of 5.6 to > 100 mg/L. The 48 h EC_{50} of the *Lupinus angustifolius* plant extract was 1.38 mg/L, which was far more toxic than the simulated extract where lethality was < 10% at 10 mg/L after 48 h. Hence, non-measured compounds may have contributed to the joint toxicity. Daphnid mothers exposed to > 2.5 mg/L sparteine produced significantly fewer and smaller offspring during the 21-day exposure, making chronic effects occur at concentrations approximately 10-fold lower than the 48 h EC_{50} for sparteine. The risk assessment of cumulated alkaloids measured in drain, running and pond waters showed a potential risk, particularly for stagnant pond water, where concentrations were severalfold higher than in the drain and running waters.

Conclusions: The results highlight that natural toxins may contribute to poor chemical quality of natural waters, and that natural toxins from upcoming crops or invasive weeds should be considered in aquatic risk assessments.

Keywords: *Daphnia magna*, Alkaloid, Plant toxins, Risk assessment, Aquatic risk assessment

Background

Many plants contain plant secondary metabolites, some of which can act as attractors of pollinators, defence against bacterial, viral and fungal diseases, and/or deterrers of herbivores. Of the 200,000 different plant secondary metabolites identified, many are phytotoxins, typically acting as an anti-herbivore defence mechanism [45]. Growing evidence suggests that some of these phytotoxins are mobile in soil, leaching from the plant to freshwater sources such as lakes, rivers and groundwater [6, 44]. This exposure may pose a risk to aquatic non-target organisms, which are often of similar phylogenetic origin as the plant herbivores.

One of the largest classes of plant secondary metabolites are the alkaloids, comprising at least 21,000 structurally diverse molecules, distributed throughout 20% of known vascular plants [47]. Alkaloids are united by the presence of a nitrogen-containing heterocyclic ring and can be broadly divided into different classes such as quinolizidine, indole, pyrrolizidine and tropane alkaloids. Quinolizidine alkaloids, e.g. lupanine, lupinine and sparteine, are found predominantly in the *Fabaceae* family,

*Correspondence: ncf@plen.ku.dk
Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark

particularly in the genus *Lupinus*. The *Lupinus* genus includes more than 400 characterised lupin species, containing over 100 identified endogenous quinolizidine alkaloids, which can make up to 5% of the plants' dry weight [46]. In addition to quinolizidine alkaloids, several lupin species, such as the yellow lupin (*Lupinus luteus*), also contain indole alkaloids e.g. gramine. Lupins are mostly herbaceous perennial plants found in lowland and montane regions [46]. Like other legumes, lupins are nitrogen fixers [28]. This, together with their tolerance of low nutrient soils, has facilitated their use as an agricultural tool to enhance soil quality and reduce disease in subsequent crops by breaking cereal-dominating crop rotations [28]. Furthermore, lupins themselves are becoming an attractive protein crop, as lupin grain is high in protein (approximately 40%) and low in oil (approximately 10%), with very little starch [38]. This unique composition makes lupins a viable alternative to soya bean used in animal feed and, to a lesser extent, food for humans [29]. As a result, lupin cultivated area and production in Europe has been increasing, from 62,710 hectares in 2000 to 274,394 hectares in 2017 [16, 29].

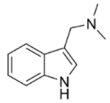
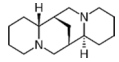
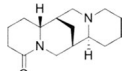
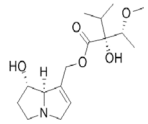
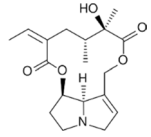
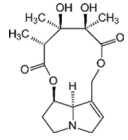
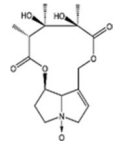
Another prevalent class of alkaloids are the pyrrolizidine alkaloids, some of which can undergo oxidation to form N-oxides. Both forms are widespread in nature, having been identified in more than 6000 plant species and being present in approximately 3% of the world's known flowering plants [7]. Pyrrolizidine alkaloids are found in at least twelve higher plant families, with the *Boraginaceae, Asteraceae (Compositae)* and *Fabaceae (Leguminosae)* families containing the most [19]. One species of *Asteraceae* known to contain pyrrolizidine alkaloids is *Senecio jacobaea* (syn. *Jacobaea vulgaris*), commonly known as the ragwort. The ragwort is a common biennial yellow wildflower native to northern Eurasia, and introduced into North America, Argentina, Australia, India, New Zealand and Northern Africa, where it is listed as a noxious weed [2]. *S. jacobaea,* along with *S. leucophyllus,* can accumulate large amounts of pyrrolizidine alkaloids, 2.2 and 4.3 mg/kg dry weight, respectively [30]. As many pyrrolizidine alkaloid-containing plants are invasive and weed-like in nature, they often dominate wild fields and agricultural land.

In conjunction with the anthropogenic and natural increase in lupin and ragwort abundance, respectively, natural stress and pest-induced damage can increase alkaloid concentrations within the plants. As seen in bracken, rain events can lead to leaching of phytotoxins into the surrounding environment [6, 26]. The toxicity of lupin plants and their isolated alkaloids is well-documented in mammals. The mode of action of many quinolizidine and indole alkaloids involves disruption to the cholinergic neurotransmission system, leading to anticholinergic effects such as impaired coordination, memory, sensory faculties, and muscular control [45]. Quinolizidine alkaloids act as acetylcholine receptor (AChR) agonists, some preferentially bind to muscarinic AChR (e.g. sparteine) while some preferentially bind to nicotinic AChR (e.g. cytisine and lupanine), with many acting at both [45]. Indole alkaloids, such as vincamine, similarly act on the central nervous system, owing to the tryptamine structural moiety found in nearly all indole alkaloids [42].

The toxicology of the pyrrolizidine alkaloids is highly dependent on their biotransformation. Hydrolysis and N-oxidation promote detoxification and excretion, hence N-oxide forms are typically less toxic than their parent compound [17]. However, oxidation via cytochrome-P450 monooxygenases can produce dehydropyrrolizidine alkaloids, highly reactive electrophilic metabolites that can interact with proteins [17]. Hepatotoxicity is the most common form of pyrrolizidine alkaloid toxicity, yet they can elicit a variety of toxicities including acute toxicity and genotoxicity such as mutagenicity, carcinogenicity, DNA cross-linking, DNA-protein cross-linking and chromosomal aberrations [17].

Considering the known toxic nature of alkaloids, together with their prevalence in plants increasingly used for agricultural purposes, it is critical to assess their potential toxicity to non-target organisms, with the aim of improving risk assessments and better managing land use. Regarding aquatic risk assessment of chemicals, e.g. pesticides (EC Regulation No. 1107/2009 [10]), biocides (EC Regulation No. 528/2012 [12]) and industrial chemicals (EC Regulation No. 1907/2006 [11]), the pelagic crustacean *Daphnia magna* is the preferred first tier organism to test. Hence, the aim of this study was to use *D. magna* to determine (1) whether the toxicity of a lupin extract can be predicted by the toxicity of its known individual alkaloids (2) whether there are differences between acute and chronic toxicity of the quinolizidine alkaloid sparteine, and (3) whether alkaloids measured in the environment may pose a potential risk to the environment. We hypothesise that toxicity of the natural mixture of alkaloids in the lupin extract can be explained by the concentration addition model, which assumes that the individual chemicals do not interact with each other [3]. Sparteine was selected to test the difference between acute and chronic toxicity, as it is found in numerous lupin species and may be considered representative due to its structure forming the base of many other quinolizidine alkaloids. Finally, we used the approach of the Water Framework Directive (EC Directive 2000/60/EC [9]) to produce environmental quality standard (EQS) values for alkaloids and applied these to concentrations measured in soil and surface waters (Hama & Strobe [20–23]).

Table 1 Summary of the properties of the selected alkaloids based on experimental data or QSAR predictions obtained from the Danish QSAR Database (http://qsar.food.dtu.dk/)

		Gramine	Sparteine	Lupinine	Lupanine	Heliotrine	Senecionine	Monocrotaline	Monocrotaline N-oxide
Type of Alkaloid		Indole	Quinolizidine	Quinolizidine	Quinolizidine	Pyrrolizidine	Pyrrolizidine	Pyrrolizidine	Pyrrolizidine
Structure									
Molecular Formula		$C_{11}H_{14}N_2$	$C_{15}H_{26}N_2$	$C_{10}H_{19}NO$	$C_{15}H_{24}N_2O$	$C_{16}H_{27}NO_5$	$C_{18}H_{25}NO_5$	$C_{16}H_{23}NO_6$	$C_{16}H_{23}NO_7$
Molecular Weight (g/mol)		174.25	234.39	169.27	248.37	313.4	335.4	325.36	341.36
Boiling Point (°C)		312.39	325[a]	270.93	374.71	394.8	503.9	502.87	*Monocrotaline N-oxide not in QSAR database*
Water Solubility (mg/L)		34,400	3,040 at 22°C[b]	2.512e + 004 at 25 °C	831.5	46,350	165,300	901,900	
pKa		8.7 (Base) n/a (Acid)	12 (Base) n/a (Acid)	9.4 (Base) 15.3 (Acid)	9.4 (Base) n/a (Acid)	9.2 (Base) 14.1 (Acid)	7.8 (Base) 12.4 (Acid)	7.8 (Base) 11.2 (Acid)	
LogKow		1.45	2.73	1.64	1.71	0.41	-0.39	− 1.18	
LogKoc (MCI method)		3.2306	3.71	1.727	3.1097	0.5358	1.9616	0.2883	
LogKoc (Kow method)		1.6618	2.304	1.355	1.7582	0.1074	0.1014	− 0.747	
Bioconcentration Factor (L/kg wet-wt)		4.218	29.3	5.609	6.271	3.162	3.162	3.162	
Level III Fugacity Environmental Partitioning $t_{1/2}$	Air	2.03 h	3.69 h	3.69 h	0.926 h	0.444 h	0.406 h	0.489 h	
	Water	60 d	37.5 d	37.5 d	37.5 d	37.5 d	37.5 d	60 d	
	Soil	120 d	75 d	75 d	75 d	75 d	75 d	120 d	
	Sediment	541.7 d	337.5 d	337.5 d	337.5 d	337.5 d	337.5 d	541.7 d	
Leadscope D. magna 48 h EC_{50} (mg/L)		5.046	3.148	13.045	3.476	254,148	3.218	9.735	

[a] PhysProp. [b] Dannenfelser and Yalkowsky [8].

Methods

Chemicals

The indole alkaloid gramine (CAS; 87-52-5, purity; 99%), and three quinolizidine alkaloids (lupanine (CAS; 550-90-3, purity; 96%), lupinine (CAS; 486-70-4, purity; 96%) and sparteine (CAS; 90-39-1, purity; 99%)) were purchased from Sigma-Aldrich (Denmark). The pyrrolizidine alkaloids were all purchased from Phytolab (Vestenbergsgreuth, Germany): heliotrine (CAS; 303-33-3, purity; 98%), monocrotaline (CAS; 315-22-0, purity; 95%), monocrotaline N-oxide (CAS; 35337-98-5, purity; 98%) and senecionine (CAS; 130-01-8, purity; 95%). A summary of the alkaloids' relevant physicochemical and environmental fate properties can be found in Table 1. The solvent methanol (CH_3OH, CAS,67-56-1, MS grade) and the internal standard caffeine ($C_8H_{10}N_4O_2$, CAS; 58-08-2, purity; 98%) were purchased from Sigma-Aldrich (Germany).

A natural mixture of alkaloids was obtained by producing a crude plant extract from the narrow-leafed lupin (*L. angustifolius)* [22]. The plant material (root, stem, leaves, flower, without the seeds) was homogenised and 150 g of this was added to 500 mL ethanol (96%) in a 1 L Erlenmeyer flask and stirred for 30 minutes at room temperature on a magnetic stirrer. The resulting supernatant was collected in another flask and this extraction process was repeated a further two times. The supernatants were pooled and then filtered through a 90 mm Frisenette 118 filter paper. A rotation evaporator was used to remove the ethanol, rotation was set at 4–5 rotations/min and the water bath temperature was set to 40 °C. The ethanol-free extract was transferred to a 100 mL Blue Cap flask with 100 mL deionised water and stored at −20 °C until needed.

D. magna culture

D. magna (F-clone), originally obtained from Barcelona, were cultured based on the OCED (Organisation for Economic Co-operation and Development) Guidelines for the Testing of Chemicals [32] The *D. magna* culture was kept in 1-L glass beakers containing approximately 0.8 L M7 medium, with 15±2 individuals per beaker, at 20 ± 2°C under a 16:8 h light:dark photoperiod, light intensity not exceeding 15–20 $\mu Em^{-2}s^{-1}$ [32]. The M7 medium was produced according to OECD [32] using salts of >98% purity purchased from Sigma-Aldrich. Organisms were fed with either the green microalgae *Raphidocelis subcapitata* or the unicellular green algae *Chlorella* (approximately 9×10^4 cells/daphnid), which were cultured based on the OECD guideline 201 for testing of chemicals [33]. All neonates used in the following tests came from this stock culture of healthy, parthenogenic reproducing mothers.

D. magna acute toxicity tests

Acute toxicity tests were performed for the crude plant extract, and technical grade gramine, heliotrine lupanine, lupinine, monocrotaline, monocrotaline N-oxide, senecionine and sparteine. To determine if any synergistic or additive relationships existed between the quinolizidine alkaloids, an acute toxicity test was also conducted for a simulated mixture, replicating the natural composition of alkaloids found in the plant extract. Due to a lack of literature values for acute toxicity of these chemicals in *D. magna* or other aquatic invertebrates, concentration ranges used for single toxicity studies were selected based on *D. magna* 48 h EC_{50} (concentration causing 50% immobility) estimates produced by quantitative structure-activity relationship (QSAR) models (Leadscope, SciQSAR and Epi ECOSAR).

The acute toxicity tests were conducted in accordance with OECD test 202 [32] and performed in 100 mL glass beakers containing 50 mL solution. In addition to the treatment groups, there were also a control group (media only) for every experiment and, where necessary, a solvent control group testing the highest solvent concentration used. The solvent limit set by the OECD guidelines is 0.01% [32]. However, as some of the individual alkaloid stock solutions were dissolved in 100% methanol, and high alkaloid concentrations were tested, solvent concentrations did occasionally exceed 0.01%. In these cases, spiked test beakers were left uncovered for 48 h to let the methanol evaporate. If no differences between control and solvent control mortality were observed, results from both groups were pooled into a singular control group.

For each treatment/control group there were four replicates, with each replicate containing five *D. magna* neonates (≤48 h old) from a healthy stock of parthenogenic mothers. The experiment was conducted under the same conditions as the stock culture was kept in. Test vessels were loosely covered with film to reduce water loss and contamination with dust or other particles. This was a static test, with no renewal of media at any point. Daphnids were not fed during the first 48 h to avoid sorption of alkaloids to food particles, but were thereafter fed approximately 9×10^4 cells/daphnid *R. subcapitata*. At 24, 48, 72 and 96 h after exposure, the number of mobile/immobile daphnids were recorded; daphnids were classed as immobile when they did not swim within 15 sec following gentle mechanical stimulation of the media.

D. magna reproductive toxicity test

The experiment, based on OECD test 211 [34], aimed to investigate the sub-lethal effects on the reproductive output of female *D. magna* over 21 days during chronic exposure to sparteine.

Neonates (≤24 h) were obtained from 4-week old parents. Each mother ($N = 10$ replicates per treatment; $N = 1$ organism per replicate) was individually exposed to 50 mL test solution in a 100 mL glass beaker in a semi-static renewal set-up for 21 d. A solvent control group was included as well as the blank control (media only). The solvent control and highest sparteine concentration group both contained 0.01% (0.1 mL/L) MeOH, which is the maximum solvent limit set by OECD [34].

Survival and reproductive output of each individual was recorded every day, with offspring gently sieved, counted and removed when present. Daphnids were fed approximately 9×10^4 cells/daphnid *R. subcapitata* daily and were transferred into clean beakers with new test solutions every third day to ensure sufficient dissolved oxygen and relatively stable sparteine concentrations. Days to production of first brood and cumulative number of neonates per female were used to assess fecundity. Only offspring produced by mothers that survived the duration of the experiment were included in the fecundity data analysis.

Once a week (days 7, 14 and 21) all mothers from each treatment group were transferred onto a plastic sheet, with another sheet gently placed on top, and subsequently scanned using a Canon CanoScan LiDE 220 A4 scanner at 1,200 DPI (dots per inch). Mothers were transferred back into their beakers immediately after scanning. The resulting images were assessed using the MeasuringBodyLength.exe software [1] in order to calculate average length, from the apex of the helmet to the base of the tail spine [49], of mothers over time for each exposure concentration. Neonates produced on days 18, 19, 20 and 21 were scanned as above.

Exposure verification

pH and oxygen

During the acute toxicity tests, pH was measured using a Metrohm® 913 pH meter (Switzerland) at 0, 48, and 96 h as minimum to check the validity of the test according to the OECD performance criteria [32]. Dissolved oxygen concentration was measured at 0, 48, and 96 h using a ProfiLine Oxi 3310 portable oxygen meter connected to an FDO® 925 oxygen sensor (WTW, Germany) to ensure dissolved oxygen concentration remained above 3 mg/L [32].

In the crude plant extract acute toxicity test, oxygen fell below 1 mg/L in all test solutions within the first 24 h. The experiment was therefore repeated with modifications; replicates were pooled ($N = 15$ daphnids per bottle) in 250 mL bottles containing 200 mL solution, and continuously aerated using an air pump (Mistral 2000, Aqua Medic GmbH, Germany) via plastic tubing and glass syringe needles. During the reproduction study, pH and dissolved oxygen concentration were measured in each new stock solution and old solutions at time of renewal.

Chemical analysis

In order to verify the exposure concentrations, samples were taken at various time points, mixed with 20 µL caffeine to a final concentration of 10 µg/L as an internal standard, and stored in amber HPLC vials at −20 °C for subsequent chemical analysis. For the acute toxicity tests, 500–1000 µL samples were taken from each beaker at 0 h before the daphnids were added, and at 48 and 96 h. For the reproduction test, 500–1000 µL samples were taken of each new stock solution at time of renewal every 3 days (days 0, 3, 6, 9, 12, 15 and 18), and of old solutions at time of renewal approximately once per week (days 3, 9, 15 and 21). The chemical analysis was carried out using Ultra Performance Liquid Chromatography (UPLC) coupled with Mass Spectrometry; UPLC-MS/MS. Details are summarized in the supplementary information (Additional file 1: Table S1), and more explicitly in [21] and [22]).

Statistical treatment of data

Toxicity data

Survival as a function of time was described by the General Unified Threshold model for Survival (GUTS) using the openGUTS software (www.openGUTS.info), using the assumption of Stocastic Death, SD, and Individual Tolerance (IT) [13], [25]. EC_{50} values were extracted for the timepoints 48 h (the standard time for acute toxicity test with *D. magna*) and 96 h (the time used in the present study).

In GUTS, the scaled damage, D_w, is modelled according to Eq. 1, where k_D is the dominant rate constant and C_W the external measured alkaloid concentration.

$$\frac{dD_w(t)}{dt} = k_D \times (C_w(t) - D_w(t)) \quad (1)$$

According to SD-theory, hazard to the organism, H, increases proportionally to the killing rate, b_w, when the scaled damage exceeds the internal threshold concentration z_w.

$$\frac{dH(t)}{dt} = b_w \times max(0, D_w(t) - z_w) \quad (2)$$

Finally, the survival probability, S_{SD}, as a function of time for an organism with an internal threshold z_w, is calculated from the hazard, H, and background hazard, h_b (control mortality).

$$S_{SD} = e^{-(H(t)+h_b(t))} \quad (3)$$

When modelling survival over time according to theory of individual tolerance, the threshold distribution, m_w, is assumed to be log-logistic and the survival probability, *F(t)*, of an individual is calculated according to a cumulative log-logistic distribution of the threshold [25].

$$F(t) = \frac{1}{1 + \left(\frac{\max_{0 \le \tau \le t} D_w(\tau)}{m_w}\right)^{-\beta}} \quad (4)$$

where m_w is the median distribution of the threshold (measured as concentration), while β is a parameter determining the width of the distribution. As survival in the IT model is related to the maximum dose metric rather than the actual dose metric until time *t*, the probability to survive (S_{IT}) can be described as:

$$S_{IT}(t) = (1 - F(t)) \times e^{-h_b \times t}. \quad (5)$$

Parameter sets for the GUTS-RED-SD and GUTS-RED-IT were obtained by minimisation of the negative log-likelihood function, and comparison of the two models was done by comparing log-likelihood values, selecting the model with the lowest value.

For the chronic toxicity study, data is presented as means and standard errors for each treatment or control group. For the time to first brood, cumulative reproduction and mother/neonate length endpoints, a One-way Analysis of Variance (ANOVA) was conducted when the data passed both a Shapiro-Wilk normality test and a Levene's homogeneity of variance test. If these parametric assumptions were not met, non-parametric statistics were conducted; in this case a Kruskal-Wallis one-way ANOVA on ranks. A Tukey HSD post-hoc test was conducted following the one-way ANOVA, or a post-hoc Dunn's test (pairwise multiple comparison) following the Kruskal-Wallis one-way ANOVA on ranks. Significance for all statistical tests was set at 0.05.

Risk assessment

To perform a tentative risk assessment of the alkaloids released from plants into the aquatic environment, four available datasets were used: two datasets of soil pore water alkaloid concentrations beneath lupin fields in Switzerland and Denmark, respectively [22, 24], and two sets of measured surface water concentrations in three Danish ponds [21, 22] and one Swiss creak surrounded by grass or meadows with alkaloid-containing plants [24]. Measured or QSAR derived daphnid toxicity data did not exist for all measured alkaloids, however, all measured and estimated 48 h EC_{50} values were within the same order of magnitude. Hence, an average acute toxicity estimate based on measured data was applied to the cumulated alkaloid concentration, and environmental quality standard (EQS) values for cumulated alkaloids were derived following the Water Framework Directive (WFD) guidance document [15]. According to the WFD, an assessment factor of 1000 is applied to acute toxicity data, typically EC_{50} values, if only data from a single or few species exists, as is the case for the present study, or an assessment factor of 100 for chronic No Observed Effect Concentration (NOEC) or EC_{10} values.

Results and discussion

Acute toxicity tests

Individual alkaloids

Initial acute toxicity tests were conducted in *D. magna* for the eight individual alkaloids. Chemical analysis of samples taken at the start of the experiment and after 96 h showed high stability over time, with > 95% of the

Table 2 The negative Log-likelihood values (LL), and parameters derived from fitting mobility of daphnids exposed to individual alkaloids and an alkaloid plant extract daily over 96 h to a GUTS-RED-IT model are given together with derived EC_{50} values asfter 48 h (standard timeframe for toxicity on *D. magna*) and 96 h

	LL	kD_IT/d	mw_IT mg/L	β	48 h EC_{50} mg/L	96 h EC_{50} mg/L
Gramine	106.6	0.411 (0.090–0.721)	3.37 (1.13–4.49)	3.07 (2.11–5.70)	6.03 (5.18–7.49)	4.19 (3.41–4.95)
Heliotrine		NA	NA	NA	> 100	> 100
Lupanine	80	0.0016 (0.0016[a]–0.173)	0.419 (0.322–33.7)	4.89 (3.02–10.0)	128 (96–185)	64 (48–93)
Lupinine	27.9	0.698 (0.002[a]–1.46)	117 (0.92–160[a])	5.10 (2.36–20[a])	156 (95–703)	125 (82–394)
Monocrotaline	37.9	0.421 (0.158–0.776)	22.4 (11.0–32.0)	2.26 (1.74–3.49)	39.4 (32.7–47.1)	27.5 (21.9–34.7)
Monocrotaline N-oxide	3.97	0.613 (0.42–6.11)	44.0 (4.05–96.8)	1.05 (1.05[a]–2.28)	62.2 (40.1–96.7)	48.1 (25.8–96.7)
Senecionine	26.3	0.969 (0.501–1.64)	45.8 (31.6–62.7)	2.85 (2.03–5.15)	53.6 (41.6–68.9)	46.8 (34.7–63.7)
Sparteine	77.3	0.0016 (0.0016[a]–0.205)	0.0937 (0.0632–9.77)	5.57 (3.12–20[a])	28.6 (18.8–40.9)	14.3 (9.4–20.7)
Plant extract	145	0.0016 (0.0016[a]–0.137)	0.0056 (0.0043–0.421)	3.91 (2.63–7.01)	1.72 (1.30–2.14)	0.86 (0.65–1.09)

All values are given with their 95% confidence intervals (CI). NA indicates non-achievable data

[a] Edge of 95% parameter CI has run into a boundary

alkaloid measured at the start of the experiment being present after 96 h for the majority of the alkaloids (Additional file 1: Table S2). The only exception was monocrotaline, where only 11% of the initial concentration was present after 96 h, possibly due to conversion to monocrotaline N-oxide. Recovery of the nominal concentration was, however, low. These low recoveries are most likely due to the long time periods (>1 year) between sampling and analytical measurements, the longest of which was for the samples from the chronic study, which had the lowest recovery. Degradation of the alkaloids during storage is therefore likely. Initial nominal concentrations are used for all toxicity estimates due to (1) the stability of the alkaloids during the experimental period, and (2) a recovery study measuring alkaloid concentrations in spiked soils and water using the same method with shorter storage time showed >90% recovery [21].

The two reduced GUTS models SD and IT generally described data equally well, with Log-Likelihood values for the estimations varying by less than 10%. As the IT model was most often the best fitting model, describing four of the seven curves that could be fitted better than the SD-model, parameter values for GUTS-RED-IT are given in Table 2 together with the EC_{50} values after 48 h and 96 h, and are used in the risk assessment. The GUTS-RED-SD parameters are given in SI together with concentration-response curves after 96 h of exposure (Additional file 1: Table S3, Figure S1). Concentration-response curves were obtained for all alkaloids except for heliotrine, where all daphnids survived at the highest concentration tested of 100 mg/L. For lupinine, only 25% of the organisms died at the highest concentration tested. Hence, these EC_{50} values should be interpreted with care. The rest of the alkaloids killed >80% of the organisms at the highest concentrations tested.

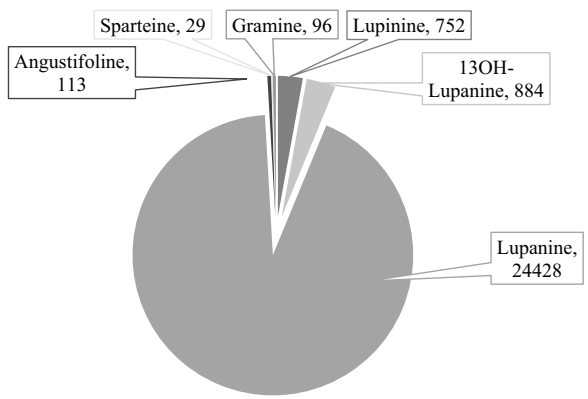


Fig. 1 Concentration of quinolizidine ($N=5$) and indole ($N=1$) alkaloids in the crude plant extract of *Lupinus angustifolius*. Values are in µg/L

Comparing experimental 48 h and 96 h EC_{50} values with 48 h EC_{50} QSAR estimates predicted using Leadscope (Leadscope Toxicity Database), Tables 1 and 2, showed that the measured EC_{50} values were <1 to 35-fold higher than the QSAR estimates for the 48 h measurements, and <1-17-fold higher for the 96 h measurements. Hence, the QSAR provided conservative QSAR estimates by always predicting similar (gramine) or more toxic measurements compared to the measured values. Hence, for risk assessment purposes, it seems the Leadscope QSAR platform estimates alkaloid toxicity towards *D. magna* within the right order of magnitude. The only other experimentally obtained 48 h EC_{50} for *D. magna* found in the literature was 12 mg/L lupanine [37], which is approximately 10-fold lower than the 128 mg/L found in this study (Table 2).

Addressing the difference in toxicity between the tested alkaloids, the indole alkaloid gramine was the most toxic (48 h EC_{50}: 6.03 mg/L), and heliotrine, lupanine and lupinine the least toxic (48 h EC_{50}: >100 mg/L), while the remaining alkaloids had toxicities in between (Table 2). There were no consistent differences in toxicities between quinolizidine and pyrrolizidine alkaloids, nor when comparisons were made on a molar basis using the molecular weights in Table 1. In mammals, lupanine and sparteine are consistently the most toxic of the quinolizidine alkaloids when administered to rats, guinea pigs and mice, with oral LD_{50} values in mice of 410 mg/kg bodyweight for lupanine and 220 mg/kg body weight for sparteine [48]. The LD_{50} values for pyrrolizidine alkaloids were within the same range when tested in rats, with LD_{50} values of 300, 175 and 85 mg/kg bodyweight for heliotrine, monocrotaline and senecionine, respectively. Hence, the two types of alkaloids seem to have comparable toxicities in mammals also.

Mixture of alkaloids

A 96 h acute toxicity test was also conducted for a crude plant extract derived from *L. angustifolius,* representing the natural mixtures of alkaloids seen in this species. As seen in Fig. 1, the plant extract consisted primarily of quinolizine alkaloids (lupanine (92.9%), 13-hydroxylupanine (3.4%), lupinine (2.9%), angustifoline (0.4%) and sparteine (0.1%)), as well as the indole alkaloid gramine (0.4%). The plant extract was 5 to 100-fold more toxic than any of the individual alkaloids tested, based on measured cumulated alkaloids, having a 96 h EC_{50} value of 860 µg/L (Table 2).

This suggests that either there are more toxic compounds in the plant extract than those measured, or the measured alkaloids interact synergistically with each other or with other plant extract components. As the concentrations of the individual alkaloids in the plant

extract were known (sum of 10 mg/L in the highest concentration), a simulated mixture was tested with similar alkaloid composition and proportion. This mixture, however, maximally killed 15% of the daphnids after 96 h, emphasising that the measured alkaloids cannot explain the observed toxicity of the plant extract, which likely stems from other unmeasured phytotoxins, alkaloid metabolites, or the enantiomeric composition of the alkaloids. The enantiomeric composition is likely different in natural extracts compared to the purchased pure compounds, with a study showing that the enantiomeric form of lupanine severely affects bioactivity [36]. Turbidity of the plant extract may be an additional factor affecting daphnid survival, as the high concentration solutions were visually green and turbid. Several studies have shown the negative influence of turbidity on daphnid behavioural responses and motility [4, 31], hence, this cannot be ruled out as a factor contributing to the observed immobility in the plant extract treatments, despite oxygen and pH being kept within the OECD limits [32].

Contrary to our study, rat studies investigating the toxicity of lupin extracts and individual, pure alkaloids, have shown mixtures to be less toxic than predicted by the toxicities of the individual alkaloids. One study exposed rats to lupanine and hydroxylupanine, which were more toxic individually than when mixed [39]. A similar study dosed rats with lupanine, resulting in an acute oral LD_{50} of 1700

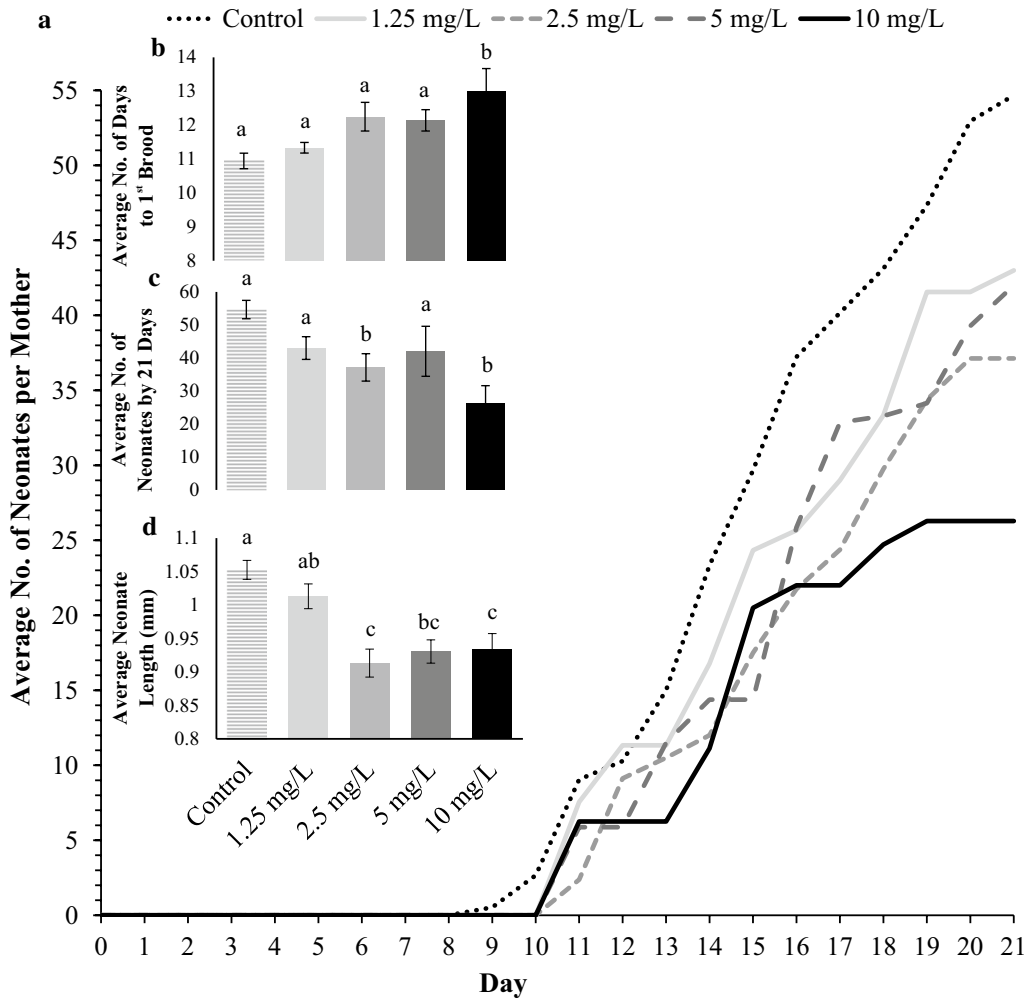


Fig. 2 Results of the chronic exposure of *D. magna* to sparteine on endpoints related to the neonates: a) Cumulative reproduction of neonates from each treatment group over the 21-day exposure period. Data is the cumulative mean number of neonates per mother for each treatment on each day; b) Mean number of days to production of first brood, ± standard error; c) Mean cumulative number of neonates per mother by 21 days, ± standard error; d) Mean length of neonates (mm), ± standard error. Small letters signify significant differences between treatments (For figure 3b Kruskal-Wallis one-way ANOVA on ranks followed by an unadjusted Dunn's test; 3c and 3d one-way ANOVA followed by a Tukey HSD test)

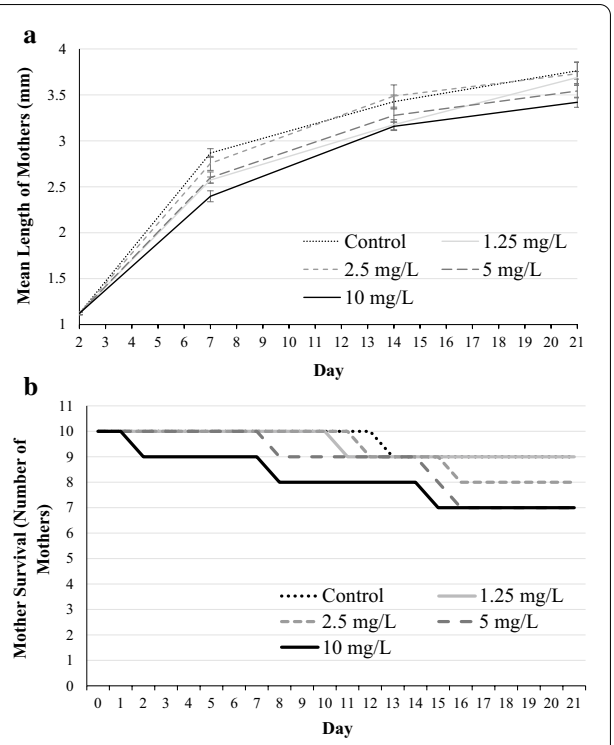


Fig. 3 Results of the chronic exposure of *D. magna* to sparteine on endpoints related to the mothers: **a** Mean mother length (mm) of daphnid mothers exposed to different sparteine concentrations, ± standard error; **b** Mother survival in each treatment group throughout the 21 days

mg/kg body weight, while an alkaloid mixture from *L. angustifolius* (49% lupanine, 39% 13-hydroxylupanine, 10% angustifoline, and 0.7% α-isolupanine) resulted in an acute oral LD_{50} of 2,300 mg/kg body weight [40].

Effects of sparteine on reproduction

While acute toxicity provides a unified 'benchmark' enabling quick comparison between different chemicals on different species, it is arguably less relevant for aquatic organisms exposed to phytotoxins, which are released over a longer period of time during the season and may even accumulate over the season due to their relative persistence [22, 23]. Therefore a 21-day sublethal exposure was conducted to investigate the reproductive toxicity of sparteine, which is considered a representative quinolizidine alkaloid. Exposure verification of sparteine concentrations at the start and end of each three-day exposure period confirmed the stability of sparteine in the test setup, with concentrations after three days being 97–117% of starting concentrations.

Sparteine concentrations corresponding to approximately 10% of the 48 h acute EC_{50} (Table 2) significantly affected growth and reproduction of daphnid mothers and their neonates (Figs. 2, 3). Time to first reproduction increased, while both the size and average number of neonates produced per mother decreased with increasing sparteine concentration (Fig. 2). The mothers grew slower with increasing sparteine concentrations, which was particularly apparent after 7 days (Fig. 3a), and there was a slightly higher mortality in the two highest concentrations (3 out of 10 mothers), compared to the low treatments and the control (1 out of 10 mothers) (Fig. 3b). All data indicate that the low chronic exposures to sparteine deprive the mother of resources. This could be either by decreasing feeding rate, as alkaloids have been shown to affect the neurotransmission system [45], or by upregulating detoxification pathways, which is an energy demanding process [41].

The delayed growth of mothers at the start of the study was also seen by Gottardi et al. [18], who exposed *D. magna* to the azole fungicide epoxiconazole and the pyrethroid insecticide α-cypermethrin for 42 days, both separately (25 mg/L epoxiconazole, or 20 ng/L α-cypermethrin) and as a mixture. They found that after 21 days there were no significant size differences between the treatments, despite the mothers exposed to α-cypermethrin or to the mixture being significantly shorter than the controls at days 3, 7 and 14. As both α-cypermethrin and sparteine affect neurotransmission, this initial reduction in mother size likely occurs by similar mechanisms, decreased feeding efficiency as a result of decreased filtration rates [5, 18]. Gottardi et al. [18] did measure P450 monooxygenase activity over time in all their treatments, but did not observe any severe upregulation.

This energetic trade-off may explain the deleterious reproductive effects of sparteine. As neonates were removed daily, i.e. within 24 h of being released from the mothers, the effects on neonate size are most likely due to the mother's sparteine exposure rather than direct exposure of neonates to sparteine in the media, although this direct short-term exposure cannot be ruled out entirely. Reduced neonate size usually leads to reduced neonate fitness; smaller neonates are e.g. three times more sensitive to acute cadmium exposure than the largest neonates [14]. However, the greater concern is the reduced cumulative reproduction and delayed production of first brood seen in the mothers exposed to 10 mg/L sparteine, as this could have population-level effects over multiple generations. Furthermore, any population-level effects of reduced reproductive rate will be exacerbated in the wild, as offspring numbers will further be reduced by predation and disease.

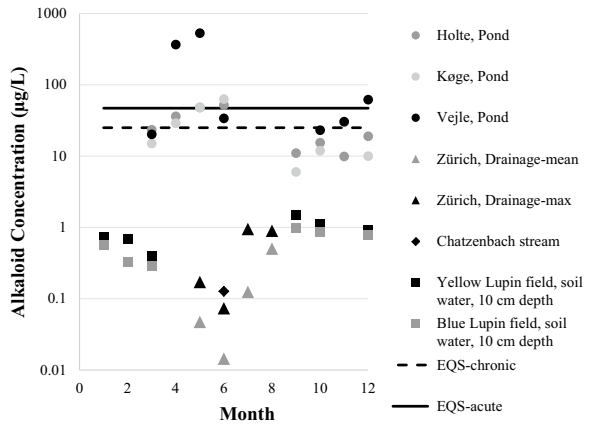


Fig. 4 Measured alkaloid concentrations in soil-, drain- and surface waters monitored in Denmark and Switzerland at different times during the year from January (month 1) to December (month 12) [23, 24]. The acute and chronic EQS values for the summed alkaloid concentrations are given by a full and broken line, respectively

Risk of lupins and other alkaloid-producing plants

Little environmental data for the concentrations of alkaloids in soil or water is currently published, however, we found four studies measuring plant alkaloids in pore, drainage and surface water [20, 21, 23, 24]. A total of 26 alkaloids were measured in these studies. Of these 26 alkaloids, we have measured daphnid toxicity values for 7, and have derived QSAR estimates for 13. Eight of the 26 measured alkaloids were N-oxide forms, which are typically less toxic than the non-oxidised form [17], as was also seen for the approximately 2-fold higher EC_{50} values of monocrotaline N-oxide compared to monocrotaline (Table 2). QSAR toxicity estimates were not possible for the N-oxide forms, or for certain alkaloids such as angustifoline, erucifoline, jacobine and jacoline.

As measured and estimated toxicity values did not exist for all alkaloids, it was decided to use average toxicity values for the 6 measured non-oxide forms or the 13 estimated non-oxide forms to derive an environmental quality standard (EQS) value. They were 69 ± 60, 47 ± 44 and 6.28 ± 4.15 mg/L for 48 h and 96 h measured EC_{50} and 48 h QSAR estimated EC_{50}, respectively. The 96 h measured average EC_{50} value was used to set the EQS as it was closer to the estimated average including twice as many alkaloids, resulting in an acute EQS of 47 µg/L, when applying an assessment factor of 1000 (European Commission, WFD). We do, however, also have a NOEC from the chronic study of 2.5 mg/L sparteine. Sparteine had a 48 h and 96 h acute EC_{50} of 28.6 and 14.3 mg/L, respectively, being in the low end of the average acute toxicity, hence it could be a reasonable conservative surrogate for the other alkaloids. Using an assessment factor of 100 applied to the NOEC, the chronic EQS is 25 µg/L. The cumulated alkaloid concentrations from the four different studies as a function of the month they were measured are shown in Fig. 4, together with the two EQS values. The graph shows that both acute and chronic EQS values are exceeded in ponds for part of the year, whereas measured soil, drainage and stream water concentrations are at least an order of magnitude below the EQS. The high pond concentrations could be due to the alkaloids accumulating in stagnant water, as they exhibit relatively high stability, pyrrolizidine alkaloids have degradation half-lives of 21 days in room temperature water and 43 days in 5 °C water [24]. Hence, stagnant waters surrounded by alkaloid-producing plants may be affected by this group of secondary metabolites.

Few studies on the risk of plant-produced alkaloids and other plant secondary metabolites exist, and for most the naturally occurring concentrations are often estimates [26, 27, 35]. Common to the saponin fraction of plant extracts, the cyclotides, and the plant-derived pharmaceutical artemisinin is that they are relatively stable under environmental conditions, with half-lives of days [26, 27, 35]. Combined with their continuous release during the season, it therefore seems that at least under some conditions, they may accumulate to toxic levels. A recent study by Tung et al. [44] researched the extremely potent group of plant secondary metabolites, aristolochic acids, which are suspected to be the cause of millions of kidney diseases and cancer cases worldwide and are persistent with no measured degradation in water over 2 months. Tung et al. [44] found these aristolochic acids leach to groundwater sources and are therefore also likely to be found in surface waters too. Their environmental risk has not been estimated, but their human toxicity indicate that aquatic fauna could be harmed.

As with any chemical, these potential risks must be weighed against benefits when it comes to regulatory decisions. The high protein, high fibre and low oil levels found in lupins, for example, makes them ideal for human and livestock consumption. Currently, most lupin production is targeted towards the feed industry, however, there is growing interest in lupins in human food products, corresponding to increasing evidence of dietary benefits [43]. Hence, more knowledge on the occurrence and toxicity of natural toxins would be beneficial when the overall risk and benefits of land management practises has to be assessed.

Conclusions

Acute toxicity to daphnids across seven quinolizidine and pyrrolizidine alkaloids varied less than 10-fold, irrespectively of alkaloid group, while the indole alkaloid gramine was more toxic than any of the other alkaloids. The chronic toxicity of sparteine was approximately 10-fold lower than acute toxicity, with the neonate growth being the most sensitive endpoint. A mixture study simulating the alkaloid composition of a plant extract showed that the analysed alkaloids could not explain the full toxicity of the plant extract, indicating that non-identified plant metabolites may account for a significant proportion of the plant extract toxicity. The present study highlights that alkaloids produced by common crops and weedy wild flora can pose a risk to the environment in line with industrial chemicals, particularly in stagnant bodies of water.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-020-00452-0.

Additional file 1: Table S1. LC-MS/MS parameters for selected alkaloids: retention time (tR), precursor ion, product ion, CVand CE. **Table S2A.** Measured concentrations of the alkaloids in the acute tests at the start of the experiment, and at the end of the 96 h experiments at a minimum of three selected concentrations. Recoveries related to the nominal concentrations are given in percent, as is the recovery of the start concentration at the end of exposure. **Table S2B.** Measured sparteine concentrations in the chronic tests at the start of each media renewal and after three days in selected treatments. Recoveries related to the nominal concentrations are given in percent, as is the recovery of the start concentration at the end of each three-day period between media renewal. Selected samples before and after the addition of food are included to investigate if sparteine sorbed to the food algae. **Table S3.** The negative Log-likelihood values (LL), and parameters derived from fitting mobility of daphnids exposed to individual alkaloids and an alkaloid plant extract daily over 96 h to a GUTS-RED-SD model are given together with derived EC50 values after 48 h (standard timeframe for toxicity on D. magna) and 96 h. All values are given with their 95% confidence intervals (CI). **Figure S1.** Concentration-response curves for the alkaloids after 96 h of exposure. $N = 4$ replicates per group; 5 daphnids per replicate. Curves obtained from R version 3.5.1.

Abbreviations

AChR: Acetylcholine receptor; ANOVA: Analysis of Variance; CI: Confidence Intervals; DPI: Dots per inch; EC_{50}: Concentration causing 50% immobility; EQS: Environmental Quality Standard; GUTS: General Unified Threshold model for Survival; LD_{50}: Concentration causing 50% lethality; LL: Log likelihood; NOEC: No Observed Effect Concentration; OECD: Organisation for Economic Co-operation and Development; QSAR: Quantitative Structure-Activity Relationship; UPLC-MS/MS: Ultra Performance Liquid Chromatography coupled with Mass Spectrometry; WFD: Water Framework Directive.

Acknowledgements

The authors wish to thank Birgitte B. Rasmussen and Anja Weibel for their technical assistance.

Authors' contributions

MG planned and conducted all toxicity tests in collaboration with NC. MG did the data treatment and wrote the first draft of the manuscript. NC supervised MG in the process, did the risk assessment analysis and assisted in writing the manuscript. JH conducted the chemical analyses under the supervision of BS. All authors read and approved the final manuscript.

Funding

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie Grant Agreement No. 722493 (NaToxAq).

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 4 November 2020 Accepted: 30 December 2020
Published online: 25 January 2021

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