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Growth strategy, phylogeny and stoichiometry determine the allelopathic potential of native and non-native plants

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Secondary compounds can contribute to the success of non-native plant species if they reduce damage by native herbivores or inhibit the growth of native plant competitors. However, there is opposing evidence on whether the secondary compounds of non-native plant species are stronger than those of natives. This may be explained by other factors, besides plant origin, that affect the potential of plant secondary compounds. We tested how plant origin, phylogeny, growth strategy and stoichiometry affected the allelopathic potential of 34 aquatic plants. The allelopathic potential was quantified using bioassays with the cyanobacterium *Dolichospermum flos-aquae*. The allelopathic potential showed a strong phylogenetic signal, but was similar for native and non-native species. Growth strategy was important, and emergent plants had twice the allelopathic potential as compared to submerged plants. Furthermore, the allelopathic potential was positively correlated to the foliar carbon-to-phosphorus (C:P) and total phenolic content. We conclude that eudicot plant species with an emergent growth strategy and high plant C:P ratio exhibit a high allelopathic potential. Unless non-native plant species match this profile, they generally have a similar allelopathic potential as natives.

Plants contain secondary compounds that improve plant fitness in various ways, for instance by reducing the growth of competitors through allelopathy (Mulderij et al. 2007, Hilt and Gross 2008, Aschehoug et al. 2014), by repelling herbivores (Callaway and Ridenour 2004, Dorenbosch and Bakker 2011, Fornoff and Gross 2014), resisting pathogens (Harborne 1977, Lattanzio et al. 2006) and interfering with decomposition (Horner et al. 1988, Bardon et al. 2014, Suseela et al. 2015, McLeod et al. 2016). Secondary compounds contribute to the success of exotic plant species, because many exotic plants possess secondary compounds that are novel to the native community (reviewed by Callaway and Ridenour 2004, Cappuccino and Carpenter 2005, Macel et al. 2014). These novel compounds are known to be bioactive (Cappuccino and Arnason 2006), and may deter native herbivores (Macel et al. 2014) or inhibit the growth of native plants (Ridenour and Callaway 2001, Svensson et al. 2013), thus improving the fitness of non-native plants, which can provide a competitive advantage over native plant

species (Callaway and Ridenour 2004, Kim and Lee 2011). However, native and non-native plants were also shown to equally deter a generalist caterpillar (Lind and Parker 2010, Grutters et al. 2016), had a similar allelopathic potential (Meiners 2014) and produced litter that was equally toxic to amphibians (Cohen et al. 2012). Thus, the activity of secondary metabolites is sometimes related to plant origin, but not always. These contrasting results may be due to natural inter- and intraspecific variation in secondary compound composition and levels, which are determined by multiple factors, including, but not limited to, plant phylogeny, plant growth strategy and elemental composition.

The secondary metabolite composition of plants is phylogenetically determined (Choi et al. 2002, Wink 2003, Gross and Bakker 2012). For example, in freshwater plants, *Myriophyllum* species contain much more phenolic compounds, specifically the deterrent tellimagrandin II, than *Potamogeton* species (Choi et al. 2002). The growth strategy of plants also affects levels of secondary compounds that are higher in forbs than in graminoids (Scharfy et al. 2011), herbaceous species are more allelopathic than woody species (Meiners 2014) and in wetlands, emergent species contain more phenolics than submerged plant species (Smolders et al. 2000). Besides phylogeny and growth strategy, the composition of plant secondary compounds may also be

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linked to relative availabilities of carbon (C), nitrogen (N) and phosphorus (P) (Bryant et al. 1985, Tuomi et al. 1988, Herms and Mattson 1992, Jones and Hartley 1999) (but see Koricheva 2002, Stamp 2003). Nutrient-limited vascular plant species were shown to contain more C-rich phenolics than nutrient replete plants (Wright et al. 2010). Phenotypic plasticity can thus affect within-species variation in phenolic compounds. Similarly, the synthesis of C-rich toxic metabolites by phytoplankton is generally enhanced under N or P limitation (Van de Waal et al. 2014).

These factors regulating secondary metabolites do not act on their own, but are interrelated. Non-native plants, for instance, often differ in phylogeny from natives, which can provide a competitive advantage (Hill and Kotanen 2009, Parker et al. 2012). Furthermore, the growth strategy of plants is generally phylogenetically determined (Scharfy et al. 2011) and the growth strategy can make certain invaders more dominant, for example forbs over graminoids, or floating over submerged plants (Scharfy et al. 2011, van Gerven et al. 2015). Although multiple factors control variation in secondary metabolites in concert, they are often studied separately.

Here, we tested whether non-native plants differ from natives in the activity of their secondary compounds, hereafter termed allelopathic potential, while accounting for effects of phylogeny, growth strategy and stoichiometry. Some freshwater plants are notorious invaders with a strong impact on native ecosystem structure and functioning (Vilà et al. 2009) and excrete secondary compounds that inhibit phytoplankton and cyanobacterial competitors (Gross 2003a), a feature that may support survival of aquatic plants in eutrophic freshwater systems (Hilt and Gross 2008). Here, we hypothesised that 1) non-native plants possess a higher allelopathic potential than native plants, 2) interspecific variation in allelopathic potential has an evolutionary origin, and 3) emergent plants possess a higher allelopathic potential than submerged species. Furthermore, we hypothesised that 4) the allelopathic potential increases with increasing plant phenolic content and carbon-to-nutrient stoichiometry.

To test these hypotheses, we quantified the allelopathic potential of 34 freshwater plants, including 17 plant species non-native to northwestern Europe, using plant extracts and agar diffusion assays with the cyanobacterium *Dolichospermum flos-aquae*. We also analysed the C, N, P and total phenolics content (TPC) of plants. TPC is the most common and widespread class of secondary compounds in aquatic plants (Lodge 1991, Smolders et al. 2000, Gross and Bakker 2012).

Material and methods

Plant species

Fresh non-apical plant material was collected for 34 plant species from which 17 were native and 17 were non-native to northwestern Europe (Table 1). The native versus non-native distinction thus refers to plant origin. The selected non-native plants have all been reported to cause economic or ecological damage (Hussner 2012). We selected a wide array of vascular aquatic plants adapted to living underwater,

or to living on permanently waterlogged soils. In addition to vascular plants, we also included one species belonging to the *Charales* and two *Salviniales* ferns to create a phylogenetically diverse selection of plant species. From the 34 plant species, 21 were harvested from 11 field sites in the Netherlands from late September to early October 2012 (see Supplementary material Appendix 1 Table A1 for coordinates). In addition, 13 plant species (...native and ... non-native) were bought from a plant breeder (Table 1). Of all species, 22 plant species were kept in a greenhouse on artificial pond sediment (Pokon Naturado, Veenendaal, the Netherlands) for a minimum of two weeks prior to collecting leaf tissue samples. This period in the greenhouse served to keep the collected plant material healthy, or to let the species grow more biomass, until they were used for analysis (see also Grutters et al. 2017a). Tissue of the other species was sampled upon collection (Table 1). Species kept in the greenhouse did not differ in morphological and physiological traits from those not kept in the greenhouse (t-tests $p > 0.05$). We classified the plant growth strategy as submerged or emergent plants, with floating-leaved plants listed as emergent because their biochemistry resembles emergent plants more than submerged ones (Smolders et al. 2000). The distribution of plant growth strategy was kept as balanced as possible: nine of the 17 native and seven of the 17 non-native plant species were submerged. The role of phylogeny was tested in two ways. In the first, plant species were grouped into monocot and eudicot for use in linear models (ANOVA). However, this excluded six species because these species could not be grouped into mono- or eudicot (Table 1). In addition, a basic monocot vs eudicot analysis may fail to capture the phylogenetic signal in secondary compounds (Zhi-Yuan et al. 2016). Therefore, all plant species were included in the second phylogenetic analysis using generalized least squares.

Chemical analyses of plant material

To analyse physiological traits, namely C, N, P content and TPC, fresh plant material was split into eight aliquots that were all thoroughly rinsed with tap water and weighed (Table 1 gives details on the sampled plant parts). Five aliquots were then oven dried (60°C for 72 h), weighed and ground with a ball grinder. The remaining three aliquotes were freeze-dried, weighed and then ground also using a ball grinder. To quantify TPC, 10 mg of oven-dried ground plant material was extracted with 5 ml of 80% EtOH for 10 min at 80°C before SDS solution and FeCl₃ reagent were added (Smolders et al. 2000). The resulting reduction of Fe³⁺ to Fe²⁺ by phenolic compounds was measured at 510 nm on a spectrophotometer against a tannic acid calibration curve and expressed as mg tannic acid equivalents per gram plant dry weight. We used oven-dried material instead of the more commonly applied freeze-dried material because insufficient freeze-dried biomass was available. We note that this may have affected the absolute levels of phenolics, but probably not the relative levels (Asami et al. 2003). To measure foliar C and N content, 1.5 mg of ground, oven-dried plant sample was weighed in tin cups and analysed using an elemental analyser. The plant P content was determined by incinerating 1 mg of ground oven-dried sample at 500°C for 30 min, followed by digestion with 5 ml of 2.5% persulphate in an

Table 1. Freshwater plant species used in the analyses. Species were classified into clades (monocot, eudicot and ‘other’ in case of taxa outside these clades, Fig. 1), plant origin described as native status to northwestern (NW) Europe, native range (ARS-GRIN database at <www.ars-grin.gov> and Hussner 2012), growth strategy (emergent and floating plants versus submerged plants), greenhouse acclimation (whether species were held in the greenhouse) and plant part (which part of the plant was sampled).

Plant species	Clade	Native to NW Europe	Growth strategy	Native range	Greenhouse acclimation	Plant part
<i>Alisma plantago-aquatica</i>	monocot	yes	emergent	holarctic	yes	leaf
<i>Callitriche platycarpa</i>	eudicot	yes	submerged	Europe, Asia	yes	shoot
<i>Ceratophyllum demersum</i>	other	yes	submerged	cosmopolitan	no	shoot
<i>Chara contraria</i>	other	yes	submerged	paleartic	no	shoot
<i>Hottonia palustris</i>	eudicot	yes	submerged	worldwide	yes	shoot
<i>Hydrocharis morsus-ranae</i>	monocot	yes	emergent	Europe, Asia	no	leaf
<i>Hydrocotyle vulgaris</i>	eudicot	yes	emergent	Europe, Asia	no	leaf
<i>Menyanthes trifoliata</i>	eudicot	yes	emergent	holarctic	yes	leaf
<i>Myriophyllum spicatum</i>	eudicot	yes	submerged	Europe, Asia	yes	shoot
<i>Nuphar lutea</i>	other	yes	emergent	Europe, Asia	no	leaf
<i>Nymphaea alba</i>	other	yes	emergent	Europe, Asia	no	leaf
<i>Potamogeton lucens</i>	monocot	yes	submerged	Europe, Asia	no	leaf
<i>Potamogeton natans</i>	monocot	yes	emergent	holarctic	yes	leaf
<i>Potamogeton perfoliatus</i>	monocot	yes	submerged	holarctic, Australia	no	leaf
<i>Potamogeton pusillus</i>	monocot	yes	submerged	worldwide	no	shoot
<i>Ranunculus circinatus</i>	eudicot	yes	submerged	Europe	no	shoot
<i>Stratiotes aloides</i>	monocot	yes	emergent	Europe	no	leaf
<i>Azolla filiculoides</i>	other	no	emergent	N-,C-,S- America	yes	leaf
<i>Crassula helmsii</i>	eudicot	no	emergent	Australasia	yes	shoot
<i>Egeria densa</i>	monocot	no	submerged	South America	yes	shoot
<i>Egeria naïas</i>	monocot	no	submerged	South America	yes	shoot
<i>Eichhornia crassipes</i>	monocot	no	emergent	South America	yes	leaf
<i>Elodea canadensis</i>	monocot	no	submerged	North America	yes	shoot
<i>Elodea nuttallii</i>	monocot	no	submerged	North America	yes	shoot
<i>Hydrocotyle ranunculoides</i>	eudicot	no	emergent	N-,C-,S- America	yes	leaf
<i>Lagarosiphon major</i>	monocot	no	submerged	South Africa	yes	shoot
<i>Ludwigia grandiflora</i>	eudicot	no	emergent	South America	yes	leaf
<i>Ludwigia peploides</i>	eudicot	no	emergent	South America	yes	leaf
<i>Lysichiton americanus</i>	monocot	no	emergent	W North America	yes	leaf
<i>Myriophyllum aquaticum</i>	eudicot	no	emergent	South America	yes	shoot
<i>Myriophyllum heterophyllum</i>	eudicot	no	submerged	SW North America	yes	shoot
<i>Pistia stratiotes</i>	monocot	no	emergent	South America	yes	leaf
<i>Salvinia molesta</i>	other	no	emergent	South America	yes	leaf
<i>Vallisneria spiralis</i>	monocot	no	submerged	North Africa, Asia, Europe	no	leaf

autoclave (30 min at 121°C). Samples were then centrifuged (30 min at 2500 rpm) and the dissolved P of the supernatant was analysed using an autoanalyzer. Total soluble sugar content (sum of sorbitol, mannitol, glucose, fructose, sucrose, maltose, trehalose, raffinose, melezitose and melibiose) was determined through liquid chromatography–tandem mass spectrometry (ultra-performance chromatographic system coupled to a tandem mass spectrometer) on the methanolic extracts of freeze-dried plant samples that were also used in the agar diffusion bioassays.

Agar diffusion assays

We performed bioassays to assess the allelopathic potential (Meiners 2014) of the entire array of chemicals produced by each plant species (Gross et al. 1991, Engel et al. 2006, Bauer et al. 2009). Bioassays provide a standardised measure of the allelopathic potential of many plant species without requiring knowledge on individual or unidentified compounds (Kubaneck et al. 2001, Engel et al. 2006, Puglisi et al. 2007). Following van Dam and Oomen (2008), freeze-dried ground plant material was extracted twice with MeOH (70% v/v). For the first extraction, the material was

boiled for 5 min and sonicated for 15 min before it was centrifuged (10 000 rpm) and the supernatant collected. For the second extraction, this procedure was repeated without boiling. The supernatants of both extractions were combined and stored at –20°C until use in the bioassays. For the agar diffusion bioassays (ADA), we cultivated the cyanobacterium *Dolichospermum flos-aquae* (formerly *Anabaena flos-aquae*; Wacklin et al. 2009) strain 141/1b in WC medium (Guillard 1975) supplemented with the vitamins B12, biotin and Thiamine HCl (Kilham et al. 1998). Liquid batch cultures were incubated at 24°C at a light intensity of 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a photoperiod of 16 h : 8 h, light : dark, in climate-controlled incubators. The agar diffusion bioassay (ADA) was performed using inoculation densities with an optical density of 0.3 at 530 nm (Gross et al. 1991, Bauer et al. 2009). A base agar of 15 ml of WC medium (1% agar) was poured into each petri dish (\varnothing 9 cm). Three aliquots (40 μl) of the methanolic extracts, which correspond to 2 mg plant dry mass, were then spotted onto the agar (Supplementary material Appendix 1 Fig. A1). After drying, the base agar was covered with 10 ml of *Dolichospermum* suspended in 1% agar, incubated at 28°C at 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for seven

days (Gross et al. 1991), and all plates were subsequently scanned to a photo using a digital scanner. Scans were then analysed to calculate the surface area in millimetres of individual clearing spots using ImageJ software. In addition to the plant extracts, we used a negative (70% aqueous MeOH v/v) and a positive control (3 μ l of 1.5% NaClO in water, w/w) in the ADAs. Negative controls did not show clearing spots, whereas positive controls showed clearing spots (average of 258 mm² per spot).

Statistical analyses

We averaged the area of all three individual clearing spots on each plate for statistical analyses. This average area is referred to as plant allelopathic potential. The variance in allelopathic potential explained by plant phylogeny, growth strategy and plant origin was tested separately using t-tests that assumed heteroscedasticity, and tested simultaneously using heteroscedasticity-corrected three-way ANOVA. In addition, we applied (multiple) linear regression to test whether plant traits explained interspecific patterns in allelopathic potential. Variance inflation factors were calculated to assess multicollinearity and we found no severe problems as the highest variance inflation factor detected, in this case for C:P, was only 6.1. We used Pearson correlation to assess the linear correlation among variables.

For phylogenetic analyses more thorough than the dichotomous classification in the ANOVA, we constructed a plant phylogenetic tree using Phylomatic (Webb and Donoghue 2005) in combination with adjustments to branch lengths using recent molecular literature [Nymphaeales (Yoo et al. 2005, Biswal et al. 2012); Hydrocharitaceae (Chen et al. 2012b); Alismataceae

(Chen et al. 2012a); Potamogetonaceae (Lindqvist et al. 2006); Salviniaceae (Metzgar et al. 2007); *Myriophyllum* (Moody and Les 2007); Lamiales (Schäferhoff et al. 2010)] and used it to calculate the phylogenetic signal in plant traits using Blomberg's K (Blomberg et al. 2003). For phylogenetic generalised least squares (PGLS) we used the Brownian model for trait evolution as correlation structure, because it fitted data better than an Ornstein–Uhlenbeck model according to AIC comparison (Burnham and Anderson 2002). We tested the significance of fixed factors in PGLS models using likelihood ratio tests.

TPC was square-root transformed to meet model assumptions. Data were analysed in R ver. 3.2.2 (<www.r-project.org>) using the *doBy* (Højsgaard et al. 2015), *nlme* (Pinheiro et al. 2015), *picante* (Kembel et al. 2010), *ape* (Paradis et al. 2004) and *car* packages (Fox and Weisberg 2011).

Data availability

Data are available from the Dryad Digital Repository: <<https://dx.doi.org/10.5061/dryad.8jc52>> (Grutters et al. 2017b).

Results

Allelopathic potential and explanatory factors

The 34 tested aquatic plant species varied considerably in their allelopathic potential (Fig. 1). Plant phylogeny, growth strategy and plant origin significantly affected the allelopathic potential when tested together in a three-way

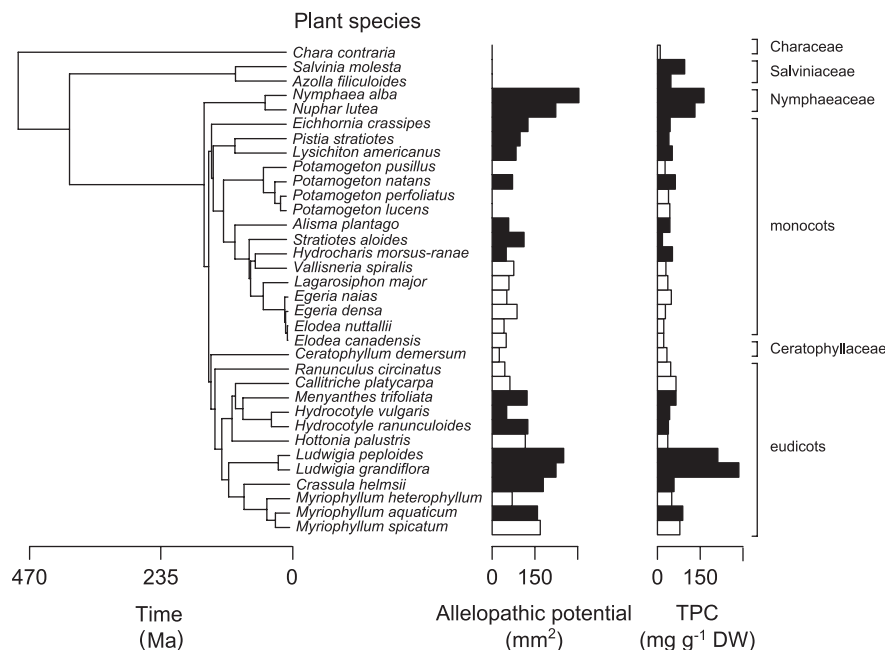


Figure 1. Phylogeny-based representation of plant allelopathic potential (clearing spot area in mm²) and total phenolics content (TPC; in mg g⁻¹ DW) of 18 emergent (black bars) and 16 submerged aquatic plants (white bars). The time since divergence (in million years ago; Ma) is shown on the left based on recent molecular clock data (Yoo et al. 2005, Lindqvist et al. 2006, Metzgar et al. 2007, Moody and Les 2007, Schäferhoff et al. 2010, Biswal et al. 2012, Chen et al. 2012a, b). The clades to which plant species belong are displayed on the right. The absence of bars indicates undetectable clearing spots (0 mm²).

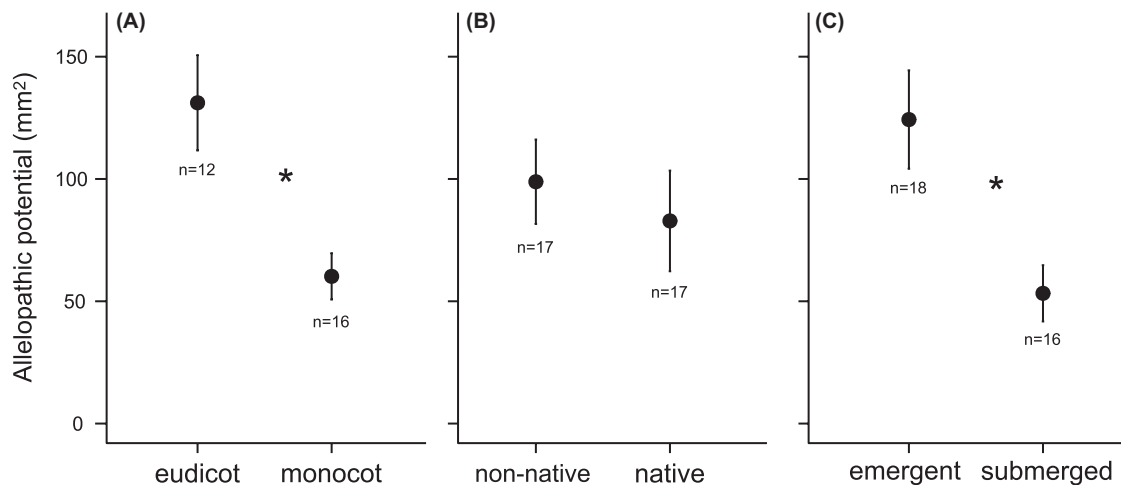


Figure 2. Mean (\pm SE) plant allelopathic potential (clearing spot area in mm²) of plant species grouped according to plant clade (A), plant origin in northwestern Europe (B) and growth strategy (C). Asterisks indicate a significant statistical difference between both means tested using t-tests assuming heteroscedasticity ($p < 0.05$). 'n' represents the number of plant species in each category.

ANOVA: plant phylogeny explained most variation in allelopathic potential ($F_{1,24} = 12.8$, $p = 0.002$), plant growth strategy explained slightly less variation ($F_{1,24} = 9.3$, $p = 0.005$) and plant origin explained least of the variation ($F_{1,24} = 8.4$, $p = 0.008$). Separate t-tests showed that eudicots exhibited a higher allelopathic potential than monocots (Fig. 2A, $t_{16,2} = 3.3$, $p = 0.005$), whereas there was no effect of plant origin: native and non-native plant species had a similar mean allelopathic potential (Fig. 2B, $t_{31,1} = 0.60$, $p = 0.56$). For growth strategy, we found that emergent plants had a higher allelopathic potential than submerged plants (Fig. 2C, $t_{26,7} = 3.1$, $p = 0.005$).

Allelopathic potential and phylogenetic analyses

There was also a strong phylogenetic signal in the allelopathic potential of aquatic plants (Fig. 1; Blomberg's $K = 0.61$,

$p = 0.001$; where a $K = 1$ represents trait evolution that is linearly proportional to plant relatedness; Grafen 1989; Table 2). Phylogenetic generalised least squares analysis (PGLS) produced results similar to linear models. Plant origin did not explain variation in allelopathic potential, whereas plant growth strategy did (two-way PGLS, growth strategy: LR = 6.8, $p = 0.009$; origin: LR = 0.22, $p = 0.64$) when tested together.

Allelopathic potential and physiological traits

Of the measured plant traits, TPC had a significant phylogenetic signal ($K = 0.50$, $p = 0.001$), whereas the signals for C content and C:P ratio were significant but much weaker (Table 2). The other traits showed no significant phylogenetic signal (Table 2). Plant traits differed most between emergent and submerged species, with TPC, C content, P content,

Table 2. Overview of plant traits grouped for growth strategy, origin and phylogeny. Means (\pm SE) of measured plant traits grouped for plant growth strategy, plant origin (native or non-native in northwestern Europe) and phylogeny. Measured traits were total phenolics content (TPC), carbon content (C), nitrogen content (N), phosphorus content (P), carbon-to-nitrogen ratio (C:N), carbon-to-phosphorus ratio (C:P) and total soluble sugars (Sugars). The phylogenetic signal displays Blomberg's K value for each of the plant traits. Values in bold indicate statistically significantly different groups, tested with t-tests assuming heteroscedasticity. 'n' indicates the number of plant species in each category.

	Growth strategy		Plant origin		Phylogeny		Phylogenetic signal	
	emergent	submerged	native	non-native	monocot	eudicot	K	p
TPC (mg g ⁻¹ DW)	n = 18 85.6 \pm 70.2	n = 16 38.5 \pm 17.1	n = 17 56.3 \pm 38.9	n = 17 70.5 \pm 71.2	n = 16 38.1 \pm 12.8	n = 12 88.9 \pm 77.9	0.50	0.001
C (% DW)	$t_{23,4} = 3.1$, $p = 0.005$ 43.9 \pm 3.1	39 \pm 4.2	$t_{31,9} = 0.56$, $p = 0.58$ 41.1 \pm 5.2	42.1 \pm 3.4	$t_{3,2} = 16.2$, $p = 0.005$ 40.6 \pm 2.9	42.9 \pm 2.9	0.36	0.036
N (% DW)	$t_{27,3} = 3.8$, $p = 0.0007$ 2.9 \pm 1.1	2.9 \pm 1	$t_{27,6} = 0.66$, $p = 0.51$ 3.1 \pm 0.8	2.8 \pm 1.2	$t_{24,0} = 2.05$, $p = 0.052$ 2.9 \pm 1	3.2 \pm 1	0.05	0.89
P (% DW)	$t_{32,0} = -0.03$, $p = 0.98$ 0.7 \pm 0.2	1.2 \pm 0.5	$t_{28,0} = -0.78$, $p = 0.44$ 0.9 \pm 0.3	1 \pm 0.5	$t_{23,8} = 0.68$, $p = 0.50$ 1 \pm 0.5	0.8 \pm 0.3	0.07	0.61
C:N (mol mol ⁻¹)	$t_{20,8} = -3.9$, $p < 0.001$ 22.1 \pm 14.6	17.3 \pm 6.3	$t_{28,2} = 0.94$, $p = 0.36$ 16.5 \pm 3.6	23.2 \pm 15.4	$t_{25,9} = -1.46$, $p = 0.16$ 20 \pm 13.6	18.3 \pm 10.1	0.14	0.48
C:P (mol mol ⁻¹)	$t_{23,7} = 1.3$, $p = 0.22$ 178 \pm 58.1	99.7 \pm 41.4	$t_{20,6} = 1.48$, $p = 0.16$ 145.5 \pm 60.4	136.8 \pm 68.9	$t_{25,7} = -0.28$, $p = 0.79$ 123.3 \pm 50.9	167.9 \pm 71.8	0.24	0.024
Sugars (mg g ⁻¹ DW)	$t_{30,7} = 4.6$, $p < 0.001$ 54.4 \pm 32.6	23.1 \pm 14.2	$t_{31,5} = -0.39$, $p = 0.70$ 43.5 \pm 39.5	35.9 \pm 15.8	$t_{18,9} = 1.83$, $p = 0.083$ 40.5 \pm 38.9	40.0 \pm 18.8	0.20	0.094
	$t_{28,0} = 4.4$, $p < 0.001$		$t_{23,7} = 0.36$, $p = 0.72$		$t_{25,7} = 0.56$, $p = 0.58$			

C:P ratio and total soluble sugar content being higher in the former (Table 2). In addition, eudicots had a higher TPC than monocots (Table 2).

TPC best explained patterns in the allelopathic potential and both showed a positive relation ($R^2 = 0.52$, $p < 0.001$; Fig. 3). The allelopathic potential also increased with the plant C:P ratio ($R^2 = 0.32$, $p < 0.001$), but was not related to the plant C:N ratio ($R^2 = -0.03$, $p = 0.95$) or sugar content ($R^2 = 0.006$, $p = 0.28$). Testing C, N and P contents separately, plant C and P contents significantly explained variation in the allelopathic potential (respectively $R^2 = 0.20$, $p = 0.005$ and $R^2 = 0.15$, $p = 0.013$), but N content did not ($R^2 = 0$, $p = 0.30$). PGLS analysis on the allelopathic potential and plant traits produced qualitatively similar results (Fig. 3), however the plant C:P ratio explained the allelopathic potential better (logLik = -181.6) than the TPC (logLik = -183.2). Separately, plant C content best explained the allelopathic potential (logLik = -182.0, $p = 0.003$), followed by P content (logLik = -184.1, $p = 0.026$) and no effect of N content (logLik = -186.2, $p = 0.41$). TPC correlated positively with C content (Pearson correlation: $r = 0.55$, $p < 0.001$) and C:P ratio ($r = 0.61$, $p < 0.001$), negatively to P content ($r = -0.39$, $p = 0.023$), and not with C:N ($r = -0.09$, $p = 0.62$). The C:N ratio of plants was strongly correlated with their N content ($r = -0.82$, $p < 0.001$), while their C:P ratio depended strongly on P content ($r = -0.86$, $p < 0.001$).

Discussion

Our results show that multiple factors control the allelopathic potential of aquatic plants, but contrary to our hypothesis, plant origin was not one of them (hypothesis 1). Instead, the allelopathic potential was primarily constrained by phylogeny (hypothesis 2). In addition, the plant growth strategy explained much of the variation in the allelopathic potential (hypothesis 3). Besides these factors, we found that two physiological traits, the C:P ratio and total phenolics content (TPC), explained interspecific patterns in the allelopathic potential (hypothesis 4). Therefore, we reject our first hypothesis, whereas we confirm the other three.

Phenolic compounds

Phenolic compounds presumably controlled the allelopathic potential to a large degree as TPC explained 52% of its interspecific variation. Phenolic compounds play a role in many ecological processes (Appel 1993, Close and McArthur 2002), including the induction of allelopathic effects against phytoplankton (Gross et al. 2007). Both the allelopathic potential and TPC showed a strong phylogenetic signal, with a higher allelopathic potential and TPC in eudicot species than monocot species, and no inhibition of the cyanobacterium in ferns and stoneworts (Fig. 1). The growth strategy of plants was also strongly related to their allelopathic potential, with emergent species having a larger potential

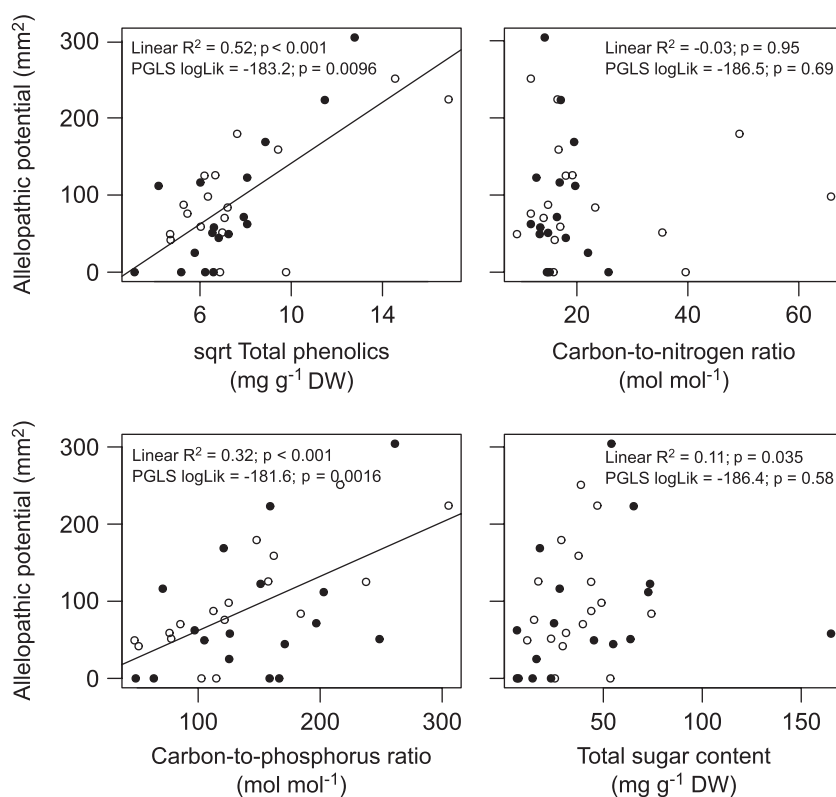


Figure 3. The relationship between the allelopathic potential of plants and four traits ($n = 34$ aquatic plant species). Regression lines are shown for significant relations and represent results of linear regression. Adjusted R^2 of linear regression and log-likelihood of phylogenetic generalised least squares (PGLS) analysis are shown along with p-values of respectively F-tests and likelihood ratio tests. Open symbols indicate non-native species, closed symbols indicate native species.

and containing more TPC than submerged plant species. It is thus conceivable that the allelopathic potential depended mainly on TPC, which is in line with the widely known ecological effects of phenolic compounds, including lowering of fungal or herbivore damage (Lodge 1991, Vergeer and Van der Velde 1997, Elger and Lemoine 2005, Lattanzio et al. 2006), slowing down microbial decomposition (Bardon et al. 2014) and inhibiting competitors through allelopathy (Gross et al. 1996). However, phenolics form a highly diverse group of compounds and are a coarse proxy for plant allelopathic effects (Leu et al. 2002, Gross 2003a). Presumably, only a subset of phenolic compounds are responsible for the observed effects (Leu et al. 2002, Sotka et al. 2009), and levels of these active compounds may differ among plant species. Because the majority of active compounds in aquatic plants are still unknown (Sotka et al. 2009, Forbey et al. 2013), we could solely use TPC as a proxy to express levels of secondary compounds in the tested aquatic plant species. Besides phenolics, freshwater plants also produce other active compounds, such as sulphuric compounds (Wium-Andersen et al. 1983) or alkaloids (Elakovich and Yang 1996). Yet, phenolics are currently the compounds most consistently related to inhibitory activity (Planas et al. 1981, Leu et al. 2002, Gross and Bakker 2012). Our results seem to further confirm that phenolic compounds play a substantial role in the allelopathic activity of aquatic plants.

The level of phenolic compounds varied greatly among plant species. Variability between replicates and sites can be due to several factors, e.g. the presence or not of vertical gradients along the shoot, as observed for *Myriophyllum spicatum* and *Potamogeton perfoliatus* (Hempel et al. 2009), or the attack of herbivores (Fornoff and Gross 2014). While multiple biotic and abiotic stressors control the intraspecific variation and phenotypic variation in phenolic compounds (Dixon and Paiva 1995), less is known about the drivers underlying interspecific variation. We confirmed that emergent plants generally contain higher levels of phenolic compounds than submerged plants (Smolders et al. 2000). One explanation is that emergent plants contain more structural polyphenols, such as lignin, than submerged plants. In addition, emergent plants may increase their TPC to protect against photodamage, as phenolics are powerful antioxidants (Close and McArthur 2002). TPC was shown to generally increase with increasing light levels (Close and McArthur 2002, Cronin and Lodge 2003, Gross 2003b). Terrestrial plants benefitted from the evolution of phenolic compounds, which act as 'UV light screens', when emerging from the aquatic environment onto the land (Gershenzon et al. 2012). Interestingly, we observed higher phenolic contents in the eudicots (Fig. 2A), a clade that is related more to terrestrial taxa than monocots of the order Alismatales (Cook 1999). Many eudicots are emergent or amphibious and seem accustomed to high light levels.

Furthermore, emergent plants have better access to light and CO₂ than submerged plants, thereby stimulating photosynthesis and increasing their primary carbon metabolism. This may stimulate the biosynthesis of phenolics, which is closely linked to carbon metabolism via the shikimate and polyketide pathways (Gershenzon et al. 2012, Cheyner et al. 2013). Indeed, the emergent plants in our study contained

more carbon and sugars than submerged plants, together with a higher phenolics content (Table 2). The synthesis of plant secondary metabolites is also regulated by the relative availability of resources as suggested by the highly debated carbon–nutrient–balance hypothesis (Bryant et al. 1983, Koricheva 2002, Stamp 2003) or the more general framework of ecological stoichiometry (Sterner and Elser 2002). Higher carbon:nutrient ratios in plants reflect higher cellular contents of C-rich biochemicals, which may also include C-rich secondary metabolites such as phenolic compounds. We indeed found positive correlations between both TPC and allelopathic potential and plant C:P ratios (but not with C:N ratios), across plant species. This may result from an enhanced allocation of carbon to phenolic compounds in plants possessing higher C:P ratios. These C:P ratios, however, differed also between plant growth strategies, and seemed higher in emergent plant species that require more carbon for structural biomass and for phenolic-based light screens (Close and McArthur 2002), but also have easier access to light and CO₂ compared to submerged species (Bornette and Puijalon 2011)(Table 1). It is thus unclear to what extent the allelopathic potential is directly related to the cellular availabilities of carbon, or whether it is indirectly coupled via distinct functional traits of emergent plants.

Allelopathic potential

Our results demonstrate substantial allelopathic effects of the tested plant species on the cyanobacterium *Dolichospermum flos-aquae*, which were strongly related to underlying plant traits. Freshwater submerged plants are known to excrete allelochemicals that inhibit the growth of a wide variety of phytoplankton and cyanobacteria species (van Donk and van de Bund 2002). Allelopathic effects can be highly species-specific, but can also be effective against multiple cyanobacteria and algae (Erhard and Gross 2006, Gross et al. 2007). Although our study demonstrates the allelopathic potential against the common cyanobacterium *D. flos-aquae*, further research should elucidate whether the allelopathic potential extends to other species of phytoplankton and cyanobacteria.

An important unanswered question is the degree to which secondary compounds are excreted in the field as allelochemicals (Hilt and Gross 2008). We assessed the effect of cell extracts, but not all compounds present in extracts may be excreted in sufficiently high concentrations (Gross et al. 2007). Some excreted compounds may be rapidly metabolised by bacteria or transformed by (UV-) light and oxygen. For some algae, the allelopathic effect of whole algal cells has been shown to be stronger than extracts (Martens et al. 2016), indicating that active compounds were rapidly excreted. Besides excretion, plants also lose compounds through leaching, which may affect cyanobacteria, phytoplankton and other biota living in the vicinity of the plants. Leaching effects of litter can be strong: phenolics leached from emergent plant litter are toxic to amphibians (Cohen et al. 2012), whereas saponins leaching from aquatic plants and tannins leaching from bark can poison fish (Applebaum and Birk 1979, Temmink et al. 1989). We assume that the cellular level of phenolic compounds is a good proxy for excreted (exuded, leached) active compounds, as several of

our tested plant species with high phenolic content possess a high allelopathic activity of exudates (Gross et al. 1996, Erhard and Gross 2006).

Secondary compounds can also affect ecosystem functioning without being excreted, because they interfere with the microbial decomposition of plants and nutrient cycling. Polyphenolics reduce nutrient cycling in terrestrial (Hättenschwiler and Vitousek 2000, Cornwell et al. 2008, Bardon et al. 2014) and wetland ecosystems (Freeman et al. 2004) by inhibiting microbial decomposition and by complexing nutrients.

Non-native plants

The scientific literature contains many opposing results on whether the strength of plant secondary compounds depends on plant native origin. Non-native plants can contain stronger or more secondary compounds than natives (Callaway and Ridenour 2004), but not in all cases (Lind and Parker 2010, Meiners 2014). We found that tested for all plant species, the allelopathic potential of aquatic plant species was unrelated to plant origin, neither when tested in combination with plant phylogeny and growth strategy nor separately. Only when tested with a subset of mono- and eudicot species, non-native plants had a slightly higher allelopathic potential than native plants on top of growth strategy and plant clade. Because plant origin appears unrelated or only weakly related to the allelopathic potential, we suggest that reports on stronger secondary metabolites in non-native plants may arise from differences with natives attributable to phylogeny and growth strategy. Our study demonstrates that invading eudicots with an emergent or floating growth strategy are most likely to possess strong allelopathic potential. Or possibly vice versa, species possessing these traits may be more likely to show invasive behaviour.

Conclusion

We show that interspecific variation in the allelopathic potential of aquatic plants depends on several components. First, plant phylogeny and growth strategy explain most variation. Second, plant stoichiometry, which also depends on the plant's growth strategy, determines how much plants can invest in secondary compounds. The plant allelopathic potential was strongly correlated with the plant's total phenolic content. Plant origin had no effect on their allelopathic potential: non-native plants contained similar levels of total phenolic compounds and had similar allelopathic potential as natives. We thus conclude that plant species with high allelopathic potential have a clear profile of being eudicots with an emergent growth strategy and high plant C:P ratio. Unless non-native plant species match this profile, they generally have similar allelopathic potential as natives.

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Supplementary material (available online as Appendix oik-03956 at <www.oikosjournal.org/appendix/oik-03956>). Appendix 1.