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Short communication

Algicidal activity and potential antifouling defenses in macroalgae from the western Antarctic Peninsula including probable synergistic effects of multiple compounds

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Abstract

Insoluble crude extract fractions of 11 species of Antarctic macroalgae were screened for algicidal activity against sympatric diatoms. Medium pressure liquid chromatography (MPLC) fractions of soluble extracts of four species were also screened. Levels of activity in insoluble fractions were lower than in previously examined corresponding soluble fractions. Combined MPLC fractions had lower activity than unfractionated extracts in three species, but in two, specific fractions with activity were identified. Combined soluble fractions of a fourth species retained strong activity but no individual fraction had activity even at tenfold higher concentrations, indicating that activity probably results from synergistic interactions between multiple compounds.

Keywords: algicide; Antarctica; biofouling; chemical defenses; diatoms; synergy.

Hard bottom communities along the western Antarctic Peninsula are dominated by dense, high biomass assemblages of macroalgae that provide food and cover for a wide variety of other organisms (Wiencke and Amsler 2012). The vast majority of the macroalgal biomass present resists direct herbivory by producing chemical defenses (Amsler et al. 2005a, 2008, 2009a). Another feature of the macroalgal assemblage is a relative lack of biofouling by microscopic or filamentous algae (Peters 2003, Amsler et al. 2009b). A primary reason for this is probably grazing by high densities of amphipods and other small herbivores commonly associated with the macroalgae (Peters 2003, Amsler et al. 2009b, Aumack et al. 2011). However, some macroalgal species remain relatively free of diatom biofouling in the absence of these grazers (Aumack et al. 2011), suggesting the possibility of macroalgal chemical defenses against diatoms in addition to those known for resistance to herbivores.

Numerous studies have reported that macroalgal extracts or specific compounds isolated from macroalgae have algicidal or other potential anti-biofouling activity *in vitro* (e.g., review by Bhadury and Wright 2004). However, *in vitro* bioactivity only indicates a potential for ecologically relevant effects since the active compounds would have to be present at the macroalgal surface and in sufficient concentrations to function as fouling defenses in nature (Steinberg and de Nys 2002). Such ecologically relevant activity has so far been demonstrated in only a small number of macroalgae (reviewed by Lane and Kubanek 2008, Amsler 2012). As a first step towards identifying compounds responsible for possible antifouling defense in Antarctic macroalgae, our research group previously reported on *in vitro* algicidal bioactivity in the seawater-soluble portions of lipophilic and hydrophilic crude extracts of 22 species of macroalgae from the western Antarctic Peninsula (Amsler et al. 2005b). Anti-diatom bioactivity was common and often strong in these extracts, and a total of eight extracts from seven species were identified as particularly interesting for further study.

The work reported herein had two goals. First, to examine the seawater insoluble components of the macroalgal crude extracts that could not be bioassayed by the technique utilized by Amsler et al. (2005b). This was done for ten of the species studied previously plus one additional species. The second goal was to begin bioassay-guided fractionation of four of the eight algicidal extracts previously identified as those most likely to contain compounds that might ultimately be found to have ecologically relevant anti-diatom bioactivity.

We dried seawater insoluble components of both lipophilic and hydrophilic crude extracts of ten macroalgal species onto plate well bottoms and bioassayed against the sympatric diatom *Syndroposis* sp. The strain utilized has also been used in several related studies with extracts of algae and invertebrates collected from the Antarctic Peninsula (McClintock et al. 2004, Amsler et al. 2005b, Peters et al. 2010, Koplovitz et al. 2011) and was chosen because small pennate diatoms are commonly observed epibionts on macroalgae and invertebrates in these communities (C. Amsler, personal observations). Only the seawater insoluble component of the lipophilic extract was assayed from *Gymnogongrus turquetii* because the hydrophilic extract was lost during preparation. Six of the 21 extracts had significant algicidal bioactivity

Table 1   Percentage of diatom cells[1] dead after 3 days exposure to insoluble macroalgal extracts[2].

	Insoluble fraction, lipophilic extract[4]				Insoluble fraction, hydrophilic extract[4]			
	Control	0.3×	1×	3×	Control	0.3×	1×	3×
Rhodophyceae[5]								
Curdiea racovitzae	0.67	2	98.33	100	0.33	0.33	0	2
Hariot	B	B	A	A	A	A	A	A
Gigartina skottsbergii	0	0	0	0.33	0.33	1.67	0.33	1
Setchell *et* Gardner	A	A	A	A	A	A	A	A
Gymnogongrus turquetii Hariot	0.33	4	7.67	22.67	n.d.	n.d.	n.d.	n.d.
	C	B	B	A				
Iridaea cordata (Turner) Bory	0.33	0.67	3	2.67	1.67	1	2.67	4.33
	A	A	A	A	A	A	A	A
Myriogramme mangini (Gain)	0	2.33	100	100	1	1.67	1.67	1.67
Skottsberg	C	B	A	A	A	A	A	A
Myriogramme smithii (Hooker f. *et*	0.67	3	2.67	4.67	0	0.67	0.33	3.33
Harvey) Kylin	A	A	A	A	B	AB	B	A
Palmaria decipiens (Reinsch)	0	1	1.33	7.33	1.67	1.33	2	0.67
R. W. Ricker	B	B	B	A	A	A	A	A
Trematocarpus antarcticus (Hariot)	0	0.67	66.33	100	1	0.33	1.67	1
Fredericq *et* R. L. Moe	B	B	A	A	A	A	A	A
Varimenia macropustulosa	0.67	1.33	2.33	4	0	1.67	1	3.67
R. L. Moe[6]	A	A	A	A	A	A	A	A
Phaeophyceae[5]								
Ascoseira mirabilis	0	3.33	3	100	0.67	0.67	4	77.67
Skottsberg	C	B	B	A	B	B	B	A
Himantothallus grandifolius (A. *et*	1	0.67	0.67	97.67	0.33	1	2.67	9.33
E. S. Gepp) Zinova	B	B	B	A	B	B	AB	A

Mean percent dead. Letters below means indicate results of statistical analyses[3]; means with the same letters are not statistically different (Tukey's *post hoc* test; p>0.05). Controls=f/2 media (McLachlan 1973) only. 0.3×, 1×, and 3× indicate the concentration of macroalgal extract compared to the "natural" concentration of the extract on a surface area basis calculated as mass of extract per unit surface area of alga. n.d. = not determined. [1]The diatom utilized in bioassays is a chain-forming strain of *Syndropsis* sp. originally isolated from shallow subtidal green algae near Palmer Station (McClintock et al. 2004). [2]Macroalgae were collected by scuba diving in mid-May 2007 and February to June 2008 at 2–40 m depth within 3.5 km of Palmer Station on Anvers Island off the western Antarctic Peninsula (64° 46′S, 64° 03′W). Freshly collected macroalgal thalli from multiple individuals per species were weighed wet and their volumes calculated based on previously determined wet weight to volume ratios (Amsler et al. 2005a). The thalli were then frozen and stored at –20°C until used for chemical extractions. To determine conversions for dry extract yield per unit algal surface area (mg extract cm^{-2}), wet weight to surface area conversions were determined from multiple (1–6) 2.1 cm diameter thallus disks cut from each of five individuals of each macroalgal species, blotted dry, and weighed. Since all of the macroalgal species used are relatively thin, blade-forming algae, the height of each disk was ignored in the mass to surface area conversion. [3]Statistical analyses were performed using SAS software. Data were arcsine (square root) transformed and subjected to Levene's test for homogeneity of variance, which tests squared deviations of scores from the group means. The transformed data were compared with one way ANOVA followed by Tukey's *post hoc* test. [4]Crude extracts were prepared as described by Amsler et al. (2000, 2005a). Thalli from multiple, haphazardly selected individuals of each species were extracted in three changes of 1:1 CH_2Cl_2:methanol (24 h per exchange), which when combined resulted in a lipophilic crude extract. This was then followed by three changes (24 h each) of 1:1 methanol:water, resulting in hydrophilic extracts. For use in insoluble crude extract bioassays, lipophilic crude extracts were resuspended in 80% methanol in seawater and insoluble components precipitated by centrifugation (Amsler et al. 2000). The supernatant was dried under reduced pressure then resuspended in 4% methanol in seawater, and the insoluble components in the suspension were precipitated by centrifugation and combined with the insoluble pellet from the first precipitation. This combined material comprised the insoluble component of the lipophilic extract that was utilized in bioassays. Hydrophilic crude extracts were similarly resuspended in 4% methanol in seawater. The insoluble components in the suspension were separated by centrifugation and served as the insoluble component of the hydrophilic extract for bioassays. [5]The bottom surface of each well acted as a substitute for a surface of each algal thallus. Seawater-insoluble components of both lipophilic and hydrophilic crude extracts were coated on the bottom of the well at the surface area-normalized "natural concentration" (1×) which assumes that the extracted compounds originated only at the macroalgal surface. Because this assumption could result in over estimation of the true distribution of compounds within a thallus, we also tested 0.3-fold (0.3×) the "natural concentration." For comparisons with previous studies, threefold (3×) the "natural concentration" was additionally tested in each assay. The control treatments in all experiments consisted of f/2 media (McLachlan 1973) in wells treated with only the solvents used to coat the extracts in the experimental wells. The sample size was three replicate wells for each concentration. Bioassays were performed using Falcon 3070 96-well, culture treated plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). In order to avoid drying of extract onto the vertical surfaces of the wells, solubilized extracts were transferred to the well in multiple aliquots that covered only the bottom (Peters et al. 2010, Koplovitz et al. 2011). Each aliquot was dried under reduced air pressure before the next aliquot was applied. Thereafter, 40 μl each of pre-chilled f/2 medium and of diatoms in f/2 media (concentrated by sedimentation in a conical centrifuge tube) were then added to each well. The cells were then incubated for 3 days at 1°C with 12:12, L:D photoperiod. Fluorescein diacetate and Evan's blue stains were used to determine diatom survival, as previously described in Amsler et al. (2000). [6]*V. macropustulosa* was incorrectly reported as *Pachymenia orbicularis* (Zanardini) Setchell *et* Gardner in Amsler et al. (2005b).

at 1× "natural concentration" (Table 1) although a majority of the diatoms were killed in only three of the 1× extracts (lipophilic extracts from *Curdiea racovitzae, Myriogramme mangini,* and *Trematocarpus antarcticus*). Only three extracts (lipophilic extracts of *G. turquetii, M. mangini,* and *Ascoseira mirabilis*) had significant activity at 0.3×, and all caused only relatively low diatom mortality. Overall, the insoluble components of hydrophilic extracts displayed less bioactivity than the insoluble components of lipophilic extracts.

Crude extract components soluble in seawater (with 2% methanol) of all the species studied here except *Palmaria decipiens* had been previously bioassayed at 0.3×, 1× and 3× concentrations with this same diatom species (Amsler et al. 2005b). A major reason for the exclusion of insoluble components from that and previous studies was that their precipitates often interfered with the staining technique used to distinguish live from dead cells. Drying them onto the bottom of the plate wells, as done here, resolved this issue and allowed those components to be examined for algicidal bioactivity as well. The data from these two studies taken together mean that all chemical components of these species' crude extracts have now been assayed. Amsler et al. (2005b) also observed relatively more algicidal activity in soluble components of the lipophilic crude extracts relative to hydrophilic extracts. However, the soluble components (Amsler et al. 2005b) were overall much more bioactive than the insoluble components assayed here, particularly at 0.3× and 1× concentrations. Comparing the results of the two studies extract by extract, in many cases there was no significant bioactivity here in extracts that were significantly bioactive in Amsler et al. (2005b). That is somewhat surprising for the lipophilic crude extracts since a large percentage of each was not soluble in seawater with 2% methanol. This may be due to the differences in presentation, with diatoms immersed in the soluble components vs. diatoms in contact only with a surface coated with the insoluble components. However, it also suggests that if in fact any of the algicidal compounds contained in the extracts have ecologically relevant anti-diatom activity in nature, it may be more likely that they are compounds released into the surface boundary layer rather than adherent to the algal surface. The only extract that had significant bioactivity here but not in the corresponding, soluble extract examined by Amsler et al. (2005b) was the 3× insoluble fraction of the *Himantothallus grandifolius* lipophilic extract.

Amsler et al. (2005b) identified soluble portions from eight crude extracts (from seven species) that had anti-diatom bioactivity at 1× but that had not been observed to have anti-herbivore activity in other studies (Amsler et al. 2005a). Although bioactive compounds in these extracts could have been selected for because of other activity such as anti-pathogen function, Amsler et al. (2005b) suggested these extracts would likely be the best candidates to potentially find algicidal compounds with ecological bioactivity specific to diatoms. Four of these extracts were fractionated by medium pressure liquid chromatography (MPLC) and bioassayed in the current study.

Table 2 Effect on diatom cells of MPLC fractions of macroalgal extracts[1].

	% Dead; All fractions combined[2]				Individual fractions[2]	
	Control	1×	3×	10×	Total	Active (10×)
Rhodophyceae						
Georgiella confluens	0.67	1	3.33	99	27	1
(Reinsch) Kylin	B	B	B	A		
Gigartina skottsbergii	1.67	1	0.33	0.33	37	n.d.
	A	A	A	A		
Trematocarpus antarcticus	0.33	99.33	99	99.67	24	0
	B	A	A	A		
Phaeophyceae						
Desmarestia antarctica	0	0.33	5	99	14	2
R.L. Moe *et* Silva[3]	B	B	B	A		

Mean percent dead. Letters below means indicate results of statistical analyses; means with the same letters are not statistically different (Tukey's test, p>0.05). Controls=f/2 media (McLachlan 1973) with 2% methanol only. 1×, 3×, and 10× indicate the concentration of the combined or individual fractions compared to the "natural" concentration of the extract on a wet weight basis calculated as mass of extract per unit wet mass of algae. n.d.=not determined. [1]All information as specified in Table 1 except where otherwise noted. Macroalgal collections were made February to April 2003. [2]Lipophilic crude extracts prepared following Amsler et al. (2005a) were fractionated by MPLC (Isco CombiFlash, Isco ReadiSep silica column; Teledyne Isco, Lincoln, NE, USA) using a linear solvent gradient of 100% hexanes to 100% EtOAc (ethyl acetate) to 20% MeOH in EtOAc over 50 min. Bioassay methods were as described previously for crude extracts presented in solution (Amsler et al. 2000, 2005b, McClintock et al. 2004). Macroalgal fraction concentrations used in bioassays were at their "natural concentration", defined as the mass of dry extract fraction per unit algal thallus on a volumetric basis. MPLC-generated fractions were diluted with f/2 and 2% methanol and then added to an equal volume (40 μl) of concentrated diatoms in f/2. Final extract concentrations were 1×, 3×, or 10× the "natural concentration" and assays were performed in Falcon 3070 96-well, culture treated plates (Becton, Dickinson and Company). The cells were then incubated with the extract fractions for 3 days at 1°C with 12:12, L:D photoperiod. Initially, all MPLC fractions were combined (essentially reconstituting the original crude extract). Thereafter, individual fractions were bioassayed as indicated by the results of the combined fractions. [3]Comparable to "first year" *D. antarctica* in Amsler et al. (2005b).

No algicidal activity was detected in bioassays with 37 combined extract fractions of *Gigartina skottsbergii* at any concentration (Table 2; $F_{3,11}$=0.75, p=0.5525). Thus, no individual fractions of *G. skottsbergii* were bioassayed. In 27 combined extract fractions of *Georgiella confluens* and 14 combined extract fractions of *Desmarestia antarctica*, significant differences between controls and combined fractions were observed ($F_{3,11}$=53.78, p<0.0001 and $F_{3,11}$=290.48, p<0.0001, respectively; Table 2). In each species, only the 10× combined extract was significantly different from controls (Table 2). Only one 10× MPLC fraction of *G. confluens* had significant bioactivity by itself (49% mortality, p<0.05). Two non-sequential MPLC fractions of *D. antarctica* had 99 or 100% mortality at 10× (p<0.05 in each), while the remaining fractions had insignificant mortality levels of 5% or less. In *Trematocarpus antarcticus*, 24 combined MPLC fractions displayed significant algicidal activity ($F_{3,11}$=426.69, p<0.0001; Table 2) with all three concentrations significantly different from controls and with almost all diatoms killed in each concentration. However, no individual fraction displayed bioactivity against diatoms higher than 5.67% mortality, even at 10× (p>0.05 in all cases).

The seawater-soluble component of lipophilic extracts of all four of these species killed 100% of diatoms at both 1× and 3× concentrations in our previous study, as did 0.3× concentrations of *Desmarestia antarctica* and *Trematocarpus antarcticus* (Amsler et al. 2005b). The combined fraction bioassays in the current study were essentially recombined crude extracts. That bioactivity in them was lost completely in *Gigartina skottsbergii* and below the 10× concentration in *Georgiella confluens* and *D. antarctica* suggests the responsible bioactive compounds were degraded during the fractionation process. Nonetheless, the observation that significant algicidal activity remained in specific fractions of *G. confluens* and *D. antarctica* extracts at 10× makes these fractions candidates for further bioassay-guided purification and compound identification. The ultimate goal would be to identify and structurally characterize metabolites responsible for the anti-diatom activity and then to look for these compounds on the surface of the living macroalgae (e.g., Dworjanyn et al. 1999, Lane et al. 2009). If they were found to be present on the surface and at concentrations sufficient for the algicidal effects observed *in vitro,* it would be likely that they play an ecologically relevant antifouling role.

In contrast, the combined extract fractions of *Trematocarpus antarcticus* retained strong bioactivity even at the 1× concentration. Either the responsible metabolites are more stable during fractionation than in the other species or they retain bioactivity at much lower concentrations (as already noted, Amsler et al. (2005b) reported 100% mortality at 0.3×, which was the lowest concentration tested). Regardless, none of the 24 individual fractions had bioactivity above baseline even at 10× concentrations. This indicates that more than one compound in combination is probably responsible for the algicidal activity and that the effect is likely to be synergistic rather than simply additive. Probable synergistic effects of multiple antifouling compounds have been reported with extract fractions of sea stars (Greer et al. 2006) and individual alkaloids

elaborated by a tunicate have been reported to have synergistic effects in controlling biomedically relevant bacteria (Rinehart et al. 1987). Otherwise, we are not aware of documented examples of synergistic effects of bioactive compounds from marine organisms. This is more likely an indication of the practical difficulties of identifying synergism rather than synergistic effects being very uncommon in nature. For example, to be able to say conclusively that synergism was occurring with bioactive compounds in *T. antarcticus*, we would potentially need to bioassay all possible combinations of the 24 extract fractions to see which combinations of two or more of the fractions restored algicidal activity. Nevertheless, synergism of multiple compounds remains the most likely explanation for the effects reported here.

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