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A comprehensive evaluation of the potential chemical defenses of antarctic ascidians against sympatric fouling microorganisms

Gil Koplovitz · James B. McClintock · Charles D. Amsler · Bill J. Baker

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Abstract The present study analyzed the bioactivity of whole body extracts from six solitary and eight colonial ascidian taxa against 20 sympatric bacterial isolates and one sympatric diatom species from the Western Antarctic Peninsula. Ascidians had crude lipophilic and hydrophilic extracts assayed against 20 bacterial strains. The lipophilic extract of one ascidian caused growth inhibition in all bacterial isolates at 3× tissue-level concentrations. The lipophilic and hydrophilic extracts were fractionated into seawater-soluble and insoluble fractions and assayed at three concentrations against a sympatric diatom species. Significant diatom mortality was detected at 3× and 1× concentrations in all but one ascidian taxon. Lipophilic fractions caused higher diatom mortality than hydrophilic extracts. The specificity of secondary metabolites against diatom fouling and the lack of activity against bacteria suggest high selective pressure for chemical defenses against diatom fouling or the potential that bacterial pathogens are controlled by the ascidian immune system.

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G. Koplovitz
Department of Biology, University of Alabama at Birmingham, 1300 University Blvd., Campbell Hall 252, Birmingham, AL 35294-1170, USA
e-mail: gilkop@gmail.com

J. B. McClintock (✉)
Department of Biology, University of Alabama at Birmingham, 1300 University Blvd., Campbell Hall 368, Birmingham, AL 35294-1170, USA
e-mail: mcclinto@uab.edu

C. D. Amsler
Department of Biology, University of Alabama at Birmingham, 1300 University Blvd., Campbell Hall 367, Birmingham, AL 35294-1170, USA

B. J. Baker
Department of Chemistry, University of South Florida, Tampa, FL 33620, USA

Introduction

In marine benthic environments, exposed undefended surfaces are subject to fouling by bacteria, algae, and macroinvertebrate larvae. Sessile marine invertebrates are particularly susceptible to epiphytic and epizootic recruitment by a variety of settling organisms (Wahl 1989). Although the fouling organisms can sometimes be beneficial to their hosts by supplying them with vitamins and nitrogenous compounds, for the most part, this interaction is harmful. Negative effects include increased hydrodynamic drag, competition for nutrients, and damage to surface tissues due to mechanical anchoring of epibionts (Wahl 1989).

Ascidians are sessile, soft bodied, filter feeding organisms that occur in solitary and colonial forms in all the world's oceans. They represent important components of food webs in a variety of benthic marine communities (Monteiro et al. 2002; McClintock et al. 2004; Tatian et al. 2005). While not as vulnerable to fouling as marine sponges, fouled ascidians can be compromised by obstruction of the incurrent and exhalant siphon or clogging of the branchial basket, both critical to respiration and feeding (Lambert 1968). Moreover, increased sedimentation and increased drag caused by heavy fouling may lead to ascidians being dislodged from sediments (Stoecker 1978). A large diversity of fouling organisms commonly occurs on the outer surfaces of ascidians, particularly solitary forms (Tatian et al. 1998). These include diatoms

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(McClintock et al. 2004), bryozoans, and hydroids (McClintock and Baker 1997), as well as bacteria, algae, and other macroinvertebrates (Bryan et al. 2003).

Chemical defenses against fouling are common in ascidians and have been examined in a variety of species (Stoecker 1978; Davis and Wright 1989; Wahl and Banaigs 1991; Teo and Ryland 1995; Bryan et al. 2003; Murugan and Ramasamy 2003; Ramasamy and Murugan 2003; McClintock et al. 2004). Additionally, antimicrobial compounds have been reported in ascidians (Azumi et al. 1990b; Tsukamoto et al. 1994; Wahl et al. 1994; Findlay and Smith 1995; Wahl 1995; Murugan and Ramasamy 2003; Ramasamy and Murugan 2003). Ascidians have also been shown to harbor bacterial communities on the surface of their tunic with epibacterial densities varying between species (Wahl et al. 1994; Wahl 1995; Schuett et al. 2005). Moreover, some ascidians host intra-cellular bacteria in a presumptive symbiotic relationship (Moss et al. 2003). For example, a symbiotic relationship has been described in several tropical colonial ascidians that host the cyanobacterium *Prochloron* sp. (Cox 1986; Hirose 2005; Yokobori et al. 2006). Some bioactive secondary metabolites that have been isolated from ascidians are thought to have a bacterial origin (Bernan 2001; Schmidt et al. 2005; Donia et al. 2006; Simmons et al. 2008). In several of these cases, specific bacteria that occur on the outer surfaces of ascidians have been shown to produce antibacterial and antifouling compounds (Holmström et al. 2002; Franks et al. 2005; Dobretsov et al. 2006). Additionally, ascidians, as members of the Phylum Chordata, possess a basic immune system, which, although primitive compared to vertebrates, is relatively advanced compared to other invertebrates. Some aspects of the ascidian immune system, such as their complement system, have a level of complexity that matches that of mammals (Fujita et al. 2004).

Ascidians are ecologically important members of Antarctic benthic communities (Ramos-Esplá et al. 2005) that often dominate biomass in shallow benthic communities along the Antarctic Peninsula (Sahade et al. 1998; Tatian et al. 1998). Marine prokaryotic communities in Antarctic Peninsular waters occur in high abundance year-round (Murray et al. 1998; Ducklow et al. 2001), while microalgae dominate during seasonal spring and summer blooms (Cerrano et al. 2004). Diatoms are one of the primary fouling organisms in Antarctic benthos (El-Sayed and Fryxell 1993). For example, Antarctic sponges have been shown to be heavily fouled seasonally by benthic diatoms (Amsler et al. 2000; Cerrano et al. 2000), sometimes with deleterious effects on the host sponge (Bavestrello et al. 2000; Cerrano et al. 2000).

The present study evaluates the potential antifouling bioactivity of secondary metabolites in tissue extracts of a suite of fourteen taxa of solitary and colonial ascidians from the Western Antarctic Peninsula against a suite of 20 sympatric bacterial isolates and the diatom *Syndroposis* sp. An evaluation of the presence of potential antifouling compounds facilitates further studies to determine whether Antarctic ascidians possess ecologically relevant concentrations of these compounds on the outer tunic or in the surface boundary layers.

Materials and methods

Field collections

Ascidians were collected by hand using SCUBA from depths ranging from 2 to 39 m from various locations within a 3.5-km radius of the US Palmer Station, located on Anvers Island along the Western Antarctic Peninsula (64°46.5′S, 64°03.3′W). In order to ensure an unbiased sample of representative species, we examined all ascidian species we encountered. Collections were made during two consecutive field seasons (February–June 2007 and March–June 2008). Freshly collected solitary and colonial ascidians were subjected to volumetric (by seawater displacement in a graduated cylinder) and wet weight determinations. Ascidians were then placed in ziplock bags with identification tags and frozen at −80°C for later preparation of crude organic extracts.

Preparation of organic extracts for antibacterial and antifouling assays

Organic extracts of ascidians were prepared using whole individuals of solitary taxa and whole colonies of colonial taxa. Extraction techniques are described in McClintock et al. (2004). Briefly, several individuals or colonies were weighed, lyophilized, and then re-weighed. The freeze-dried tissues were then extracted thrice in dichloromethane/methanol (1:1 ratio) for 24 h. Extracts were combined and filtered through a coarse filter paper and dried down under reduced pressure to yield a lipophilic extract. A hydrophilic extract was prepared by subsequent extraction of the same freeze-dried tissue using methanol/water (1:1 ratio) thrice for 24 h. Both lipophilic and hydrophilic extracts were weighed following drying.

Antibacterial assays

The marine bacteria used in the present study were collected and identified in a previous study using techniques described in Peters et al. (2009). In brief, bacteria were collected from surfaces of sympatric benthic marine invertebrates by scraping their surfaces with a sterile scalpel and transferring the collected material into both

100% Difco marine broth 2216 (Difco Laboratories, Sparks, MD, USA) and 50% glycerol in marine broth using aseptic technique. The samples were then frozen at no greater than −70°C and shipped back to the United States. Frozen samples were thawed, emulsified, and incubated on marine agar to facilitate the isolation of individual bacterial colonies. The isolated colonies were grown on Difco marine agar 2216 at 4°C. Isolates were identified by sequencing their 16S rRNA gene using an ABI Prism® 3100 DNA Sequencer (AME Bioscience, Toroed, Norway). Marine broth was then inoculated with pure cultures and incubated on a shaker at 4°C until the bacteria reached the stationary phase. A 100-µl suspension of each bacterial culture was spread on marine agar plates and allowed to soak in for five min before adding each extract.

Extracts from the 14 ascidian taxa were resuspended in either methanol (for lipophilic extract) or 1:1 methanol/water (for hydrophilic extracts) at 1 ml per 3 g wet tissue originally extracted (representing 3× natural concentration). Paper antimicrobial assay disks, 6 mm diameter (BBL Microbiology Systems 31039, Cockeysville, MD, USA), were prepared by adding 20 µl (10 µl per side) of the extract solutions or of solvent only to the disks. Once the solvent had evaporated from the disks, they were placed onto the inoculated marine agar plates and incubated at 4°C for several days until bacterial growth was visible and zones of growth inhibition could be measured. If activity was detected at 3× concentration, the assay was performed again with the affected strain with disks containing 1× natural concentration. Antibacterial activity was defined as zones of inhibition around disks containing extracts when compared to solvent control disks. Each assay was replicated on three separate days with three separate bacterial cultures of each strain for a total of 3 replicates per extract concentration.

Antidiatom assay

In order to estimate the natural concentrations of secondary metabolites found on the surface of the ascidians where diatoms would be present, 1 cm × 1 cm surface squares of dried ascidians were cut with a single-edge razor blade down to a depth of approximately 0.5 mm and then weighed, approximating the dry weight of the outermost 0.5 mm of a 1 cm^2 ascidian surface. An average of the weights of available ascidian was used for all ascidians (mean = 0.032 g dry wt, range = 0.012–0.092 g dry wt). This representative weight was then used to calculate the estimated yield of lipophilic extract per surface area of an ascidian on a wet weight basis. As there is no known method of measuring the exact concentration of the organic compounds on the outer surface, we assumed that extracts were evenly distributed throughout the ascidian. In order to account for variation in the distribution of organic compounds on the surfaces of different ascidians, we bracketed the concentrations or extracts tested (see below).

The diatom that was used in the antifoulant assays was *Syndroposis* sp., a sympatric chain-forming pennate diatom previously isolated from the intertidal green alga *Cladophora repens* near Palmer Station. Diatoms were maintained in f/2 media (McLachlan 1973) at the University of Alabama at Birmingham and designated Pal D1.2, as in previous publications (McClintock et al. 2004; Amsler et al. 2005).

In order to determine which compounds (in terms of polarity) are more likely to serve as antifoulants, the lipophilic and hydrophilic extracts of the 14 ascidian taxa were further fractionated. Lipophilic extracts were resuspended in 8:2 MeOH/seawater and centrifuged (in glass vials at 1,500×*g*) to produce a soluble and an insoluble fraction. The soluble fraction was then dried and resuspended in seawater and centrifuged again to separate remaining seawater-insoluble compounds. Both insoluble fractions were combined, dried, and resuspended in 1:1 CH_2Cl_2/MeOH. The hydrophilic extracts were resuspended in seawater and centrifuged (in plastic Eppendorf tubes at 16,000×*g*) to produce a soluble and an insoluble fraction. The insoluble fraction was dried and then resuspended in MeOH.

Due to the high reactivity of CH_2Cl_2 with plastic, experiments using the insoluble fraction of the lipophilic extracts were conducted in a borosilicate glass flat-bottomed 96-well tissue culture plate (Zinsser Analytic, Frankfurt, Germany). The rest of the fractions were tested in a standard Falcon® 96-well tissue culture plate (Becton–Dickinson, Franklin Lakes, NJ, USA). For the insoluble fractions (from the lipophilic and hydrophilic extracts), the yield of extract per surface area of the ascidian was used to determine the amount of extract needed for each 6-mm diameter well (for hydrophilic extract) and 7.5 mm well (glass plate, for lipophilic extract) to approximate the natural surface concentration found in each ascidian. This concentration, as well as 30% and three times the natural concentration were determined and used in the bioassays. The solubilized extract fractions were then transferred to the wells. In order to ensure the extracts coated only the bottom surface of each well, the extracts were transferred in aliquots that covered only the bottom of the well. The plates with the extracts were then dried under reduced pressure, and subsequent aliquots were added until the appropriate amount of crude extract coated the surface of each well. Solvent control wells were made using the same method using only the appropriate solvents (1:1 CH_2Cl_2/MeOH for the lipophilic extract and MeOH for the hydrophilic extract). For the insoluble fraction of the hydrophilic extract, we initially used both solvent and non-solvent controls. After several assays, we saw no difference

between controls and subsequently used only non-solvent controls. The plate was then chilled and 40 µl of seawater was added to each well, along with 40 µl of concentrated diatoms in f/2 media. Non-solvent control wells contained only 40 µl of seawater and 40 µl of concentrated diatoms in f/2 media. The yields of the seawater-soluble fractions of the lipophilic and hydrophilic extracts were calculated on a volumetric basis as mass of extract per unit volume. Solubilized fractions were then diluted with seawater, 40 µl of the solutions were added to each well, followed by 40 µl of concentrated diatoms in f/2 media for a total concentration of 80 µl. This resulted in final concentrations of 0.3×, 1×, and 3× natural concentration on a volumetric basis.

The plates were then incubated at 1.5°C (±0.5°C) for 3 days with a 12:12 h light/dark photoperiod at an irradiance of 25 µmol photons m^{-2} s^{-1}. After incubation, the diatoms were stained with fluorescein diacetate and Evans Blue following the procedures described in Amsler et al. (2000). Thereafter, the cells were observed at 400× magnification under epiflourescence and bright field illumination on a compound microscope. Under blue epiillumination, live cells appeared green due to the fluorescein diacetate, and under bright field illumination, dead cells appeared blue due to the Evans blue staining. To calculate percent dead, a minimum of 100 cells from each replicate well were haphazardly chosen and recorded as live or dead.

Statistical analyses were performed using SigmaPlot 11.0 (SPSS Incorporated, Chicago, IL). Percentages of dead cells were arcsine (square-root) transformed. The data were then subjected to Levene's test of equality of variance. All the variances were equal; therefore, the data were subjected to one-way ANOVA followed by Tukey's post hoc tests. In some cases, where all three replicates in all three treatments were 100% dead and all controls were 0% dead, post hoc analysis was inappropriate. In these instances, treatments were designated as not significantly different from one another. Mean percent mortality for each concentration of each fraction was compared between solitary and colonial ascidian taxa using a Mann–Whitney U test (Zar 2009).

Results

Antibacterial assays

Fourteen ascidian taxa had lipophilic and hydrophilic extract assayed against 16 strains of Gamma Proteobacteria, 1 Flavobacterium, and 3 unidentified species of bacteria isolated from sympatric invertebrates in the study area. The bacterial species, strains, and number of isolates used are available in Peters et al. (2009). Of the 14 taxa of ascidians that were tested against the 20 strains of sympatric bacteria, only the lipophilic extract of one ascidian species—the colonial ascidian *Distaplia colligans*—showed broad spectrum antimicrobial activity against all bacterial strains at three times the natural concentrations (Fig. 1).

At natural concentration, the lipophilic extract of *D. colligans* caused growth inhibition (average: 0.3 mm, range: 0.2–0.4 mm) in one strain of Gamma Proteobacterium (P22—*Psychrobacter fozii*). The hydrophilic extract of *D. colligans* caused partial growth inhibition (0.5 mm zone of inhibition with sporadic colonies) in 3 bacterial strains (P22—*Psychrobacter fozii*, P29—*Alteromonas elyakovii* and P34—*Psychrobacter fozii*) in one replicate only.

Antifouling assays

Fourteen ascidian taxa had seawater-soluble and insoluble fractions of both lipophilic and hydrophilic extracts assayed at three concentrations (0.3×, 1×, and 3× estimated natural concentrations) against the sympatric chain-forming pennate diatom *Syndroposis* sp.

The seawater-insoluble fraction of the natural concentration of crude lipophilic extract caused significant (ANOVA, $P < 0.05$) diatom mortality in 11 out of 14 ascidian taxa when compared to controls (Fig. 2). At 3× natural concentration, significant diatom mortality was detected in 13 out of 14 taxa. Even at 0.3× natural concentration, eight taxa had fractions that caused significantly higher diatom mortality when compared to controls. The seawater-soluble fraction of the crude lipophilic extract caused significant (ANOVA, $P < 0.05$) diatom mortality in 10 out of 14 taxa (Fig. 3). At 3× natural concentration, significant diatom mortality was detected in 13 out of 14 taxa. At 0.3× natural concentration, 7 out of 14 taxa had fractions with significantly higher diatom mortality compared to control.

The seawater-insoluble fraction of the crude hydrophilic extract caused significant diatom mortality in five taxa (Fig. 4). However, mean diatom mortality was generally low. At 3× natural concentration, significant diatom mortality was detected in four taxa. At 0.3× natural concentration, only the extracts of 2 ascidian taxa caused significantly higher diatom mortality when compared to controls. The seawater-soluble fraction of the crude hydrophilic extract caused significant (ANOVA, $P < 0.05$) diatom mortality in extracts of only 2 species of *Distaplia* (Fig. 5). At 3× natural concentration, significant diatom mortality was detected in nine out of fourteen taxa, whereas at 0.3× natural concentration, significant diatom mortality was only detected in a single species—the colonial ascidian *Distaplia colligans*.

The seawater-soluble fraction of the hydrophilic extract of *Distaplia colligans* proved to be highly acidic, with pH values of 1.9, 3.0 and 4.2 (measured with standard analytical pH strips—EM ColorpHast) at the 3×, 1×, and

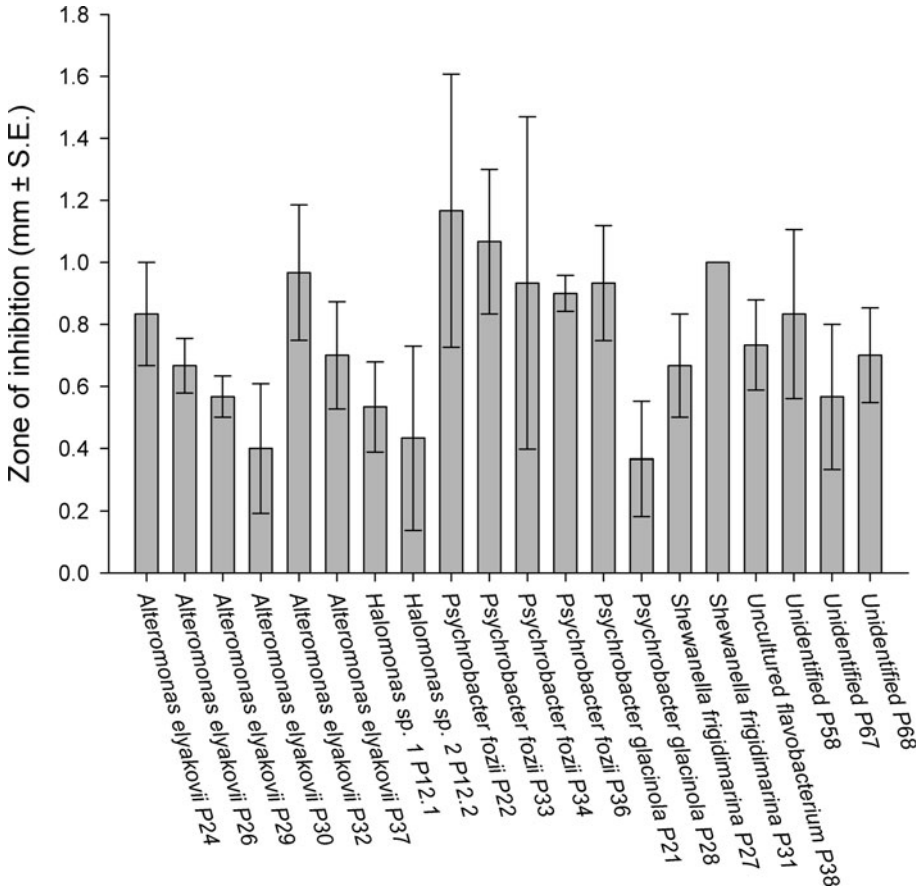

Fig. 1 *Distaplia colligans*. Results of antibacterial bioassay with lipophilic extract at 3× estimated natural concentration. There was no activity in the control disks

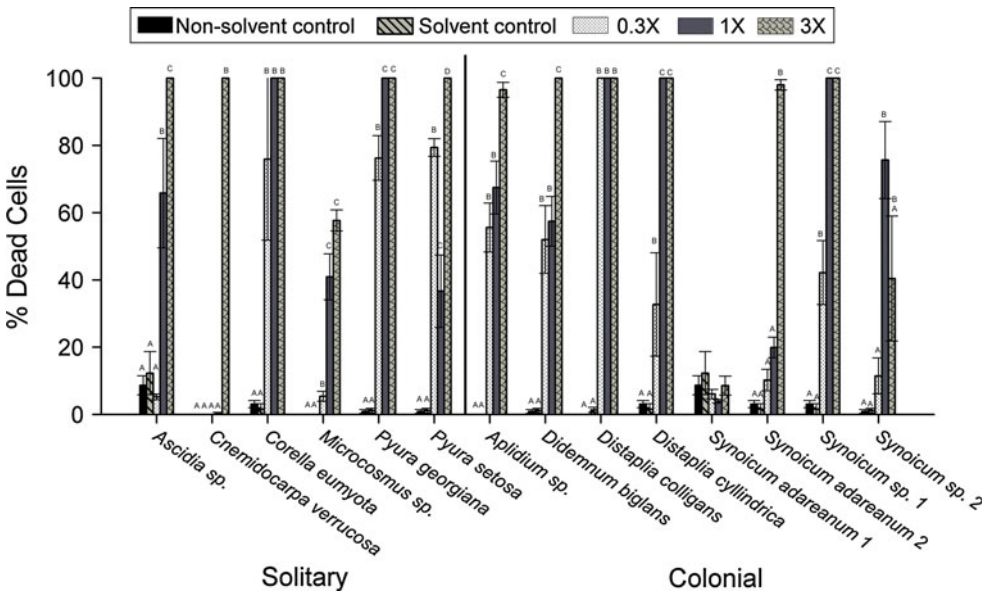

Fig. 2 Percent dead diatoms using three concentrations of seawater-insoluble fraction of lipophilic crude extracts. Shown are mean (±1 SE) percentage of dead cells ($n = 3$). Non-solvent control consisted of diatoms incubated in seawater and f/2 medium. Solvent control consisted of 1:1 dichloromethane/methanol, which was dispensed on bottom of well in aliquots and allowed to evaporate before dispensing seawater and diatoms in f/2 medium. One-way analysis of variance followed by Tukey's test was used to compare the different treatments from each species to both controls. *Bars* with *different letters* are significantly different from one another ($P \leq 0.05$)

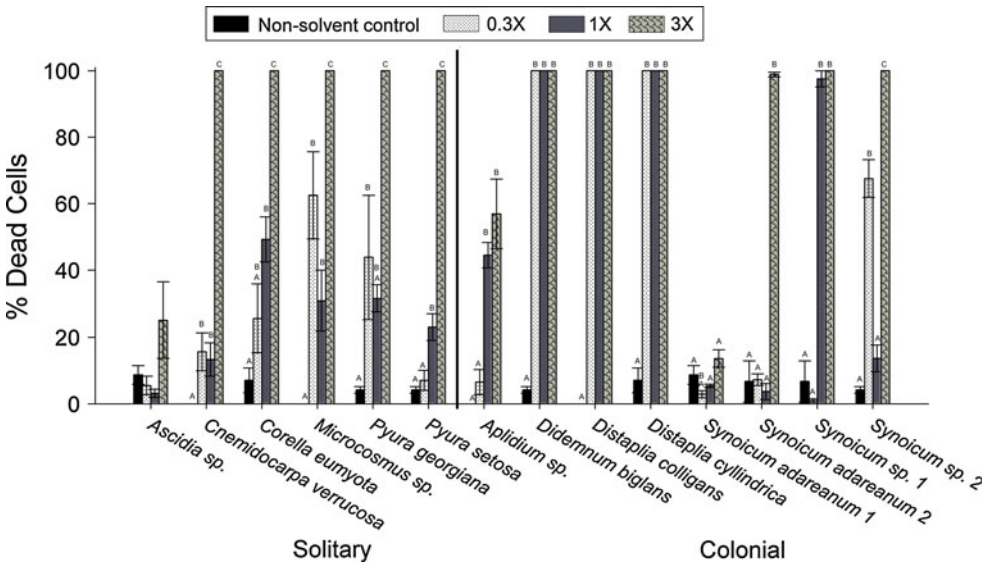

Fig. 3 Percent dead diatoms using three concentrations of seawater-soluble fraction of lipophilic crude extracts. Shown are mean (±1 SE) percentage of dead cells ($n = 3$). Non-solvent control consisted of diatoms incubated in seawater and f/2 medium. Extracts were resuspended in seawater; therefore, no solvent was used. One-way analysis of variance followed by Tukey's test was used to compare the different treatments from each species to both controls. *Bars* with *different letters* are significantly different from one another ($P \leq 0.05$)

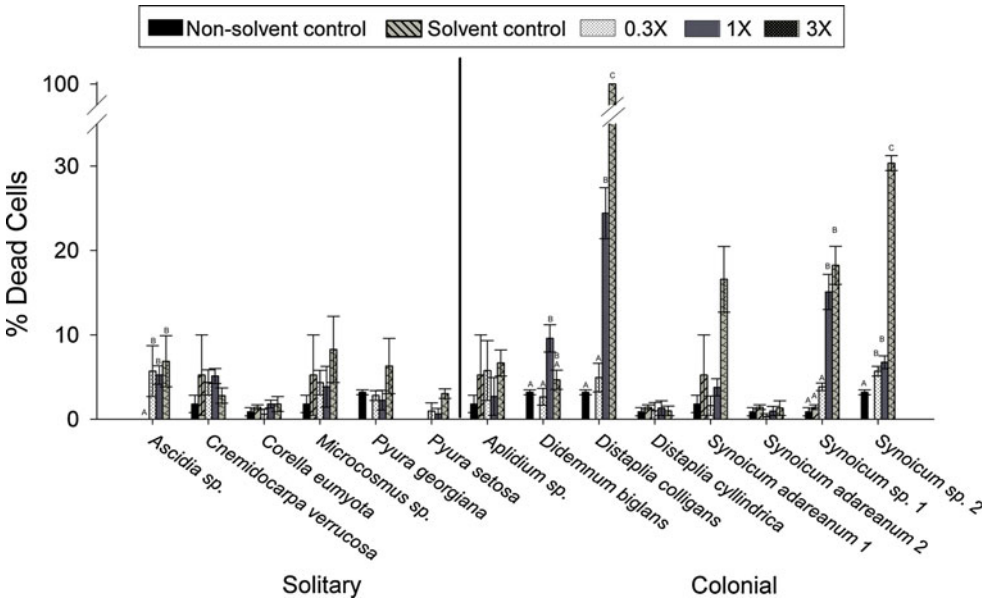

Fig. 4 Percent dead diatoms using three concentrations of seawater-insoluble fraction of hydrophilic crude extracts. Shown are mean (±1 SE) percentage of dead cells ($n = 3$). Non-solvent control consisted of diatoms incubated in seawater and f/2 medium. Solvent control consisted of methanol, which was dispensed on bottom of well in aliquots and allowed to evaporate before dispensing seawater and diatoms in f/2 medium. One-way analysis of variance followed by Tukey's test was used to compare the different treatments from each species to both controls. *Bars* with *different letters* are significantly different from one another ($P \leq 0.05$)

0.3× concentrations, respectively. The low pH interfered with the live–dead staining, mainly with the Evans Blue, which appeared yellow, but due to the appearance of the diatoms compared to the controls, all three concentrations were deemed to cause 100% diatom mortality.

The comparison of mean percent diatom mortality between extracts from the six solitary and eight colonial ascidian taxa yielded no significant differences in bioactivity among all concentrations and all fractions.

Discussion

Chemical defenses against biofouling in marine organisms have been reported from a wide variety of taxa including bacteria, algae, and a wide variety of invertebrates (e.g., Davis et al. 1989; Wahl 1989; Steinberg et al. 2002 for review). The control of epibiosis may be mediated by nonpolar compounds that are present on the outermost layer of an organism's surface or in the boundary layer, or more polar compounds that spread beyond the boundary layer (Steinberg and de Nys 2002; Steinberg et al. 2002). Whereas the more polar compounds that leach into and beyond the boundary layer might serve in repelling or preventing the settlement of fouling organisms (Walters et al. 1996; Bryan et al. 2003), in the present study, we chose to concentrate on the growth inhibition of bacteria and the antifouling activity in post-settlement diatoms. There are several limitations that arise when trying to evaluate ecologically relevant concentrations of bioactive

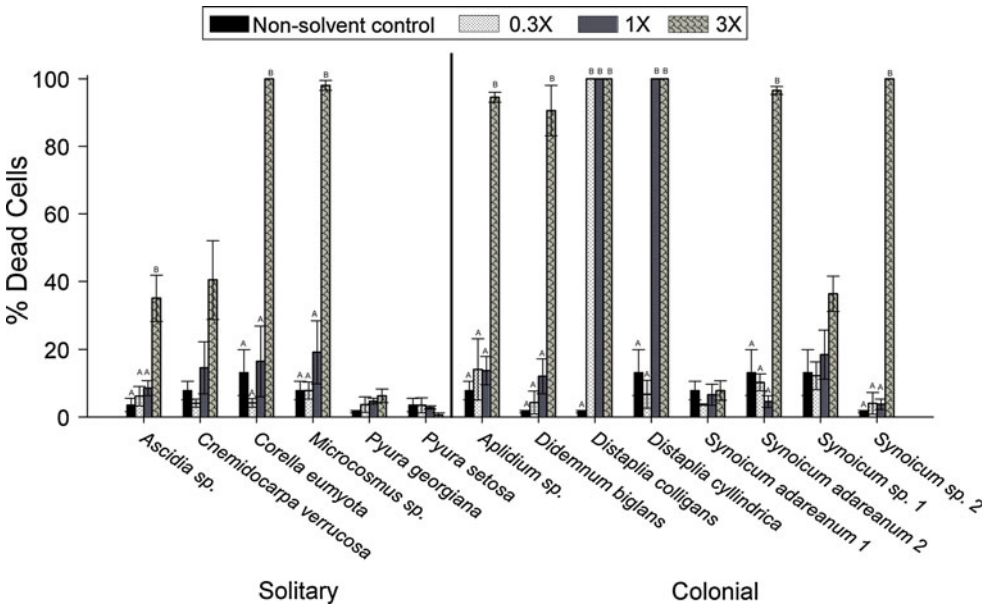

Fig. 5 Percent dead diatoms using three concentrations of seawater-soluble fraction of hydrophilic crude extracts. Shown are mean (±1 SE) percentage of dead cells ($n = 3$). Non-solvent control consisted of diatoms incubated in seawater and f/2 medium. Extracts were resuspended in seawater; therefore, no solvent was used. One-way analysis of variance followed by Tukey's test was used to compare the different treatments from each species to both controls. *Bars* with *different letters* are significantly different from one another ($P \leq 0.05$)

organic compounds that inhibit fouling microorganisms (see Amsler et al. 2000). The localization of secondary metabolites varies between ascidians (Pisut and Pawlik 2002; Salomon and Faulkner 2002; Seleghim et al. 2007), and it is unknown whether antifouling compounds are present throughout the tunic or concentrated in only the outmost layer. The Optimal Defense Theory predicts organisms will allocate defenses to areas that are most vital in terms of fitness (Rhoades 1979). Therefore, in ascidians, antifoulant metabolites are unlikely to be localized within internal tissues such as the viscera or the gonads. As the nonpolar compounds will only be encountered on the surface, for the antidiatom assays, we attempted to estimate the natural concentration based on surface area, while assuming such antifouling compounds will be concentrated on the topmost layer. For the polar compounds, as well as for the antibacterial assays, we used volumetric concentrations. In order to account for variability in the distribution of the compounds on the ascidian body, we also assayed using 30% (0.3×) and 300% (3×) of the estimated natural concentrations.

For the antibacterial assays, we initially assayed the lipophilic and hydrophilic extracts at 3× estimated natural concentration. Even so, out of 14 ascidian taxa tested, bacterial growth inhibition was detected only with the lipophilic extract of the colonial *Distaplia colligans*. This growth inhibition activity was broad spectrum affecting all 20 microbial isolates. At the estimated natural concentration, there was minor growth inhibition detected in only one of the bacterial isolates. The marine bacteria employed in the present study were isolated from a variety of sympatric benthic marine invertebrates, and it is unknown whether any are pathogens to ascidians. Peters et al. (2009) assayed lipophilic and hydrophilic extracts from 25 Antarctic sponges collected near Palmer Station against the same bacterial strains and found only one strain (*Alteromonas elyakovii*—P37) was inhibited by extracts from all 25 sponges, and four additional bacterial strains displayed sporadic grown inhibition in response to some sponge extracts. The lack of bacterial growth inhibition to extracts of 13 out of 14 ascidian taxa, including those that showed sensitivity to all 25 sponge extracts (Peters et al. 2009) suggests that Antarctic ascidians may not be prone to bacterial pathogens. Alternatively, Antarctic ascidians may be susceptible to bacterial pathogens, but have not evolved to create antibacterial compounds. A number of studies have examined the effects of ascidian secondary metabolites on human pathogenic bacteria (Azumi et al. 1990a, b; Raub et al. 1992; Tsukamoto et al. 1994; Findlay and Smith 1995). More recently, the ecological roles of antibacterial compounds have received attention (Engel et al. 2002; Bryan et al. 2003; Murugan and Ramasamy 2003; Ramasamy and Murugan 2003). Wahl et al. (1994) examined the antifouling and antimicrobial activities of secondary metabolites from a suite of temperate ascidians and found that while settlement inhibition was positively correlated with epibacterial abundance, antimicrobial activity was not. This suggests ascidians use a variety of mechanisms to control bacterial epibiosis. Ascidians, due to their taxonomic position as protochordates, possess unique immune characteristics not commonly found in other invertebrates. Although they lack a vertebrate-like adaptive immune system, ascidians have a sophisticated lectin-complement innate immune system coupled with phagocytes that enables ascidians to control bacterial and other pathogens (Cooper and Nicolò 2001; Fujita et al.

2004; Hirose 2009). These phagocytes are a type of ascidian circulating hemocyte that recognizes and ingests nonself cells as well as release opsonic agglutinins to fight foreign invaders (Ballarin et al. 2001; Hirose 2009). Morula cells are another type of common circulating hemocyte seen in ascidians and are similarly capable of encapsulating foreign bodies, as well as release phenoloxidase and other antibacterial substances (Azumi et al. 1990b; Ballarin et al. 2001; Hirose 2009). As such, some ascidians such as the Antarctic species examined in the present study may rely on these immune properties as a targeted defense against bacterial pathogens, rather than on the constitutive production of antibacterial chemical defenses. As epibacterial abundances on ascidians were not examined in the present study, we are unable to evaluate whether ascidians along the Western Antarctic Peninsula use non-chemical immune mechanisms to control bacterial epibiosis, or alternately, allow bacteria to settle upon the outer surfaces, whether as commensal or mutualistic symbionts.

The present study shows that the bioactivity of ascidian secondary metabolites against diatom fouling is substantial compared to that of antimicrobial activity. Unlike sponges, whose ostia can become clogged during diatom blooms (Barthel and Wolfrath 1989; Peters et al. 2009), ascidians are able to prevent clogging by actively closing the inhalant aperture or by squirting water and particles from the branchial sac through the apertures (Hoyle 1953). However, similar behaviors have not yet been recorded in Antarctic ascidians (Kowalke 1999), which may suggest Antarctic ascidians use means other than the physical expulsion of diatoms to prevent clogging. There has been at least one report of ascidians harboring diatoms and other eukaryotic algae within the tunic, with no apparent detrimental effects (Lambert et al. 1996). Heavy diatom spring blooms have been observed to obstruct the siphons and clog the branchial basket in the solitary ascidian *Corella willmeriana*, potentially contributing to mortality (Lambert 1968).

Unlike the antimicrobial assays, which were performed on agar plates, the antidiatom assays were performed in seawater, more closely representing the environment the diatoms encounter in nature. We therefore fractionated the lipophilic and hydrophilic extracts into seawater-soluble and seawater-insoluble fractions in order to narrow down and target the more bioactive fractions. At both 0.3× and 1× natural concentrations, antifoulant activity against the diatom *Syndroposis* sp. was observed in at least one fraction, in twelve out of fourteen ascidian taxa, and in thirteen out of fourteen ascidians at 3× natural concentration. In the latter, complete diatom mortality was observed in all solitary ascidians and in six out of eight taxa of colonial ascidians.

The colonial ascidian *Synoicum adareanum* occurs in two different morphs near Palmer Station (Authors' personal observation). One morph occurs as a large, bulbous colony (*Synoicum adareanum* 1) and the other morph occurs as several lobes attached at the base (*Synoicum adareanum* 2). Whereas all four fractions of *S. adareanum* 1 showed no bioactivity against diatoms, *S. adareanum* 2 caused significant diatom mortality at 3× natural concentration in both lipophilic fractions as well as the most polar hydrophilic fraction. This suggests a need for further taxonomic resolution in this species.

Extracts of the colonial ascidian *Distaplia colligans* displayed consistently high bioactivity with complete diatom mortality even at 0.3× natural concentration in three of four fractions. The most polar fraction also exhibited a low pH that may have contributed to diatom mortality. A similar high acidity in the organic extract of this species was observed in a previous study (Koplovitz et al. 2009). In this study, *D. colligans* was found to be the only ascidian among the same suite of taxa examined in the present study to have an organic extract that deterred feeding in the sympatric omnivorous sea star *Odontaster validus* and fish *Notothenia coriiceps*. In the present study, *D. colligans* was the only taxon to exhibit antimicrobial activity. Therefore, the organic compounds from Antarctic ascidians appear to have broad bioactivity against diatom fouling, but little to no antimicrobial or antipredatory activity. Bandurraga and Fenical (1985) isolated secondary metabolites from the pacific coral *Muricea fruticosa* and also found activity against only diatoms, with no measurable cytotoxic, ichthyotoxic, or antimicrobial activity. Evidence of antidiatom specificity was also seen in organic extracts of sponges from McMurdo Sound, Antarctica (Amsler et al. 2000).

Overall, antidiatom bioactivity in both the seawater-soluble and seawater-insoluble fractions of lipophilic extracts was higher than in the hydrophilic fractions. These fractions contain the most nonpolar compounds, which are likely to remain in, or close to, the boundary layer or on the surface of the tunic where they are effective as antifoulants (Steinberg and de Nys 2002). A similar pattern is seen in Antarctic macroalgae (Amsler et al. 2005) and Antarctic sponges (Peters et al. 2009). The seawater-soluble fraction of the hydrophilic extract (i.e., containing the most polar compounds), caused significant diatom mortality in nine out of fourteen ascidian taxa but only at 3× natural concentration. The exceptions were in *Distaplia colligans*, where 100% mortality occurred at all concentrations tested and in *Distaplia cylindrica* where 100% mortality occurred at 1× natural concentration. The percentages of the hydrophilic extracts that were insoluble in seawater was generally very low (mean: 3.58%, range: 1.66–8.31%) and the bioactivity of these fractions generally low as well. One exception was in *Distaplia colligans* where 100% diatom mortality occurred at 3× natural concentration.

Five of the ascidian taxa examined in the present study exhibited low pH on the surfaces of their tunics (Koplovitz et al. 2009). Parry (1984) suggested that tunic surface acidity may serve to prevent fouling. However, he also observed several species of non-acid-producing stolidobranch ascidians that were free of epibionts and speculated that surface acidity could not be solely responsible for ascidian antifoulant defenses. Stoecker (1978) suggested that high vanadium concentration, as well as outer surface acids enable the solitary ascidian *Ascidia nigra* to remain epibiont-free. In a survey of 35 solitary and colonial ascidians from Bermuda, Stoecker (1980) found that twelve colonial ascidians and one solitary ascidian, with high surface acidity (pH < 2), all lacked macroscopic epibionts on the tunic surface. Davis and Wright (1989) examined two colonial ascidians from Florida and found that *Eudistoma capsulatum,* which had high surface acidity (pH $= 1$–2), was heavily fouled by marine invertebrates, while its non-acidic (pH $= 6$) congener *Eudistoma olivaceum* was almost epibiont-free. They concluded that surface acidity was an ineffective defense against fouling or overgrowth by sessile invertebrates in the field. While we did not examine the effects of surface acidity or the concentration of vanadium on fouling in the present study, it is worth noting that the extracts of *Distaplia colligans* contained organic acids with a low pH. Moreover, Lebar et al. (2011) found that *D. cylindrica* had high vanadium concentrations in the body tissues.

The high incidence of bioactivity of secondary metabolites against diatom fouling in Antarctic ascidians is indicative of their possible role as antifoulants. However, the ecological relevance of such bioactivity does not always correlate with in situ observations of fouling patterns. For example, both colonial *Distaplia* species are free of fouling organisms, yet the solitary *Pyura setosa*, whose tissue extracts exhibited high diatom mortality in bioassays, are heavily fouled (Authors' personal observations). As such, it is likely that in select species, surface concentrations of bioactive compounds are insufficient to control fouling and/or are produced for other purposes. Some planktonic diatom species are known to produce toxic compounds that impair reproduction and embryonic development in copepods and other marine invertebrates (Miralto et al. 1999; Caldwell et al. 2002; Ianora et al. 2004). It is therefore possible that some ascidians allow diatoms to settle on their outer surfaces as a chemical defense mechanism in a similar fashion to ascidian epibiotic bacteria that produce antifouling compounds (Holmström et al. 2002; Franks et al. 2005; Dobretsov et al. 2006).

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