

Seaweed allelopathy to corals: are active compounds on, or in, seaweeds?

G. O. Longo^{1,2} · M. E. Hay¹

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Abstract Numerous seaweeds produce secondary metabolites that are allelopathic to corals. To date, most of the compounds identified in this interaction are lipid-soluble instead of water-soluble. Thus, understanding whether these compounds are stored internally where they would not contact corals, or occur on external surfaces where they could be transferred to corals, is critical to understanding seaweed–coral interactions and to informing realistic experiments on chemically mediated interactions. We conducted field experiments assessing the effects of lipid-soluble extracts from macroalgal surfaces alone versus total lipid-soluble extracts from both internal and external tissues on the coral *Pocillopora verrucosa*. Extracts of the red algae *Amansia rhodantha* and *Asparagopsis taxiformis*, the green alga *Chlorodesmis fastigiata*, and the brown alga *Dictyota bartayresiana* suppressed coral photochemical efficiency; in these bioactive species, the total lipid-soluble extracts were not more potent than surface-only extracts despite the concentration of total extracts being many times greater than surface-only extracts. This suggests that previous assays with total extracts may be ecologically meaningful, but also that future assays should be conducted with the simpler, less concentrated, and more ecologically relevant surface extracts. Allelopathic effects of *As. taxiformis* and *C.*

fastigiata were significantly greater than the effect of *D. bartayresiana*, with effects of *Am. rhodantha* intermediate between these groups. Neither surface-only nor total lipid-soluble extracts of the seaweed *Turbinaria ornata* were allelopathic, and its lack of potency differed significantly from all other species. Our results suggest that lipid-soluble, allelopathic compounds are usually deployed on seaweed surfaces where they can be effective in surface-mediated interactions against other species.

Keywords Seaweed–coral competition · Chemical ecology · Coral bleaching · *Pocillopora* · Moorea · South Pacific

Introduction

Secondary metabolites mediate critical ecological interactions of terrestrial and marine plants including herbivory, fouling, and allelopathy (Steinberg and de Nys 2002; Hay 2009; LoPresti 2015). The nature of the ecological interaction may select for the quantity, quality, and location of the active compounds, with location more critical for some types of interactions, such as those at surfaces (Hay 1996; Steinberg and de Nys 2002; LoPresti 2015). Herbivory, for example, can be effectively deterred by natural concentrations of compounds that are stored within plant tissues because herbivores contact these compounds upon biting and ingestion (e.g., Hay 1996; Pereira et al. 2003). Conversely, for interactions that depend on either physical proximity or direct surface contact, such as fouling or allelopathy, defensive compounds should be more effective if deployed on the plant surface where interacting organisms first come into contact (Steinberg and de Nys 2002; Nylund et al. 2007; Andras et al. 2012).

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✉ M. E. Hay
mark.hay@biology.gatech.edu

¹ School of Biological Sciences and Aquatic Chemical Ecology Center, Georgia Institute of Technology, 950 Atlantic Drive, Atlanta, GA 30332, USA

² Present Address: Department of Oceanography and Limnology, Universidade Federal do Rio Grande do Norte, Av. Via Costeira S/N, Natal, RN 59014-002, Brazil

Seaweed–coral competition is increasingly common in coral reefs worldwide, particularly where important herbivores have been overfished and no longer control seaweeds (McCook et al. 2001; Bruno et al. 2009; Hughes et al. 2010; Bonaldo and Hay 2014). Seaweeds compete with corals via multiple mechanisms (McCook et al. 2001), but allelopathy is common for numerous coral–seaweed pairings, causing coral stress, bleaching, or tissue death upon contact (e.g., Rasher and Hay 2010; Rasher et al. 2011; Andras et al. 2012; Morrow et al. 2012). Most evidence of allelopathy against adult corals comes from studies of lipid-soluble secondary metabolites that appear to function via contact rather than via dissolution in the water (Rasher and Hay 2010; Rasher et al. 2011; Andras et al. 2012). If this is a general trend, then allelopathic metabolites should be selectively deployed on seaweed surfaces where transfer from seaweed to coral would be most effective. Alternatively, more polar (water-soluble) metabolites may disrupt beneficial coral microbiomes and potentially lead to coral stress or disease (Krediet et al. 2013) under conditions where flow does not advect compounds away (Jorissen et al. 2016) or where microbial metabolism does not immediately remove them (Haas et al. 2013).

Few studies have quantified the natural concentration of chemical compounds on plant surfaces or explored the ecological differences between surface-associated compounds and those held within plant tissues even though spatial allocation could substantially change the interaction outcome (but see Steinberg and de Nys 2002; Nylund et al. 2007; Andras et al. 2012; LoPresti 2015). If compounds occurring *in* seaweeds are extracted and tested as if they occurred *on* seaweeds, then their true ecological effects may be misrepresented. For example, natural concentrations of elatol obtained from total lipid-soluble extracts of *Laurencia obtusa* significantly suppressed herbivory and fouling whereas surface concentrations did not (Pereira et al. 2003; Sudatti et al. 2008). In seaweeds, bioactive compounds may be stored in internal structures and not deployed to surfaces, may be produced in internal glands but exuded onto external surfaces via connecting pores, or may be allocated to surfaces via unknown routes (de Nys et al. 1998; Dworjanyan et al. 1999; Paul et al. 2006; Lane et al. 2009; Andras et al. 2012). Because herbivores may feed on entire thalli, a hydrophobic compound *in* the seaweed may be as ecologically important as one *on* the seaweed, but for that same compound to function as an antifouling agent, it should be on the seaweed surface where it would be encountered by the larvae of settling organisms (Schmitt et al. 1995; Nylund et al. 2007; Lane et al. 2009). Understanding where compounds occur, and in what concentrations, can inform options for their ecological function and is critical for designing ecologically realistic experiments (de Nys et al. 1998; Lane et al. 2009; Andras et al. 2012).

Seaweed allelopathy against corals can be a good model for understanding chemically mediated biotic interactions and the importance of compound location or deployment strategy. Seaweed–coral interactions can be dependent on direct contact (Rasher and Hay 2010; Rasher et al. 2011; Andras et al. 2012; Morrow et al. 2012), vary widely in consequences for different pairings of seaweed and coral species (McCook et al. 2001; Rasher et al. 2011; Bonaldo and Hay 2014), are frequent on reefs worldwide (Barott et al. 2011; Bonaldo and Hay 2014; Longo and Hay 2015), and are of increasing ecological relevance given the high frequency of phase-shifts from coral to seaweed-dominated states and the lack of recovery seen on many reefs (Bruno et al. 2009; Hughes et al. 2010; Rasher et al. 2013).

In this study, we used lipid-soluble extracts from seaweed surfaces versus entire thalli to conduct *in situ* bioassays with the common coral *Pocillopora verrucosa*. Our goals were to test the allelopathic effects of extracts from algal surfaces versus entire thalli, and to compare the allelopathic potency of different seaweed species.

Materials and methods

We tested the allelopathic potency of lipid-soluble extracts from seaweed surfaces versus entire thalli of five common seaweeds (the red algae *Amansia rhodantha* and *Asparagopsis taxiformis*, the green alga *Chlorodesmis fastigiata*, and the brown algae *Dictyota bartayresiana* and *Turbinaria ornata*) against the coral *P. verrucosa* via *in situ* bioassays on reefs of Moorea, French Polynesia. These are among the most common seaweeds in the back reef where seaweeds are abundant; the coral is also abundant in the back reef and dominates the fore reef where it constitutes the majority of coral cover (Edmunds 2012).

Seaweeds were collected from the back reef (17°47'S, 149°83'W) at depths of 1–2 m. Bioassays on corals were conducted on the fore reef (17°47'S, 149°81'W) at depths of 9–13 m where coral colonies were abundant and not in contact with macroalgae. We conducted our assays using *in situ* fore-reef corals instead of back-reef corals to prevent variance due to existing contact between back-reef corals and various seaweeds. Macroalgae were rare on the fore reef, allowing us to use corals without variable, and unknown, histories of macroalgal contact.

For extractions, we used a 20-mL volume of each seaweed species determined by volumetric displacement in seawater after being spun in a salad spinner to remove excess water. Seaweeds for surface-only and entire extracts were collected at the same time from the same site, but extractions were performed separately on different sub-samples of the collection. Lipid-soluble

surface extracts were obtained by placing the 20-mL volume of each seaweed in 100 mL of hexanes, shaking vigorously for 30 s, and immediately removing the seaweed to avoid cell lysis (de Nys et al. 1998). This extract was dried by rotary evaporation and stored in a $-20\text{ }^{\circ}\text{C}$ freezer. Previous experiments demonstrated that hexanes do not mix with water, penetrate wet cells, or cause cell lysis when applied for 30 s with several seaweed species including *C. fastigiata* (de Nys et al. 1998; Rasher and Hay 2010); thus, hexanes acquire lipid-soluble metabolites from seaweed surfaces but not from within wet cells. The efficiency of this procedure is unknown, but efficiency of hexanes using damp algae is likely less than 100%.

To obtain total lipid-soluble extracts, a 20-mL volume of each wet seaweed was extracted twice with 100 mL of methanol, dried using rotary evaporation, re-dissolved in water and ethyl acetate, and partitioned in a separator funnel. Water-soluble materials were discarded and the ethyl acetate (lipid-soluble) portion was dried by rotary evaporation and stored at $-20\text{ }^{\circ}\text{C}$ for $<24\text{ h}$ until used in field bioassays (Rasher and Hay 2010; Rasher et al. 2011). Methanol mixes well with water, penetrates wet cells, and extracts a greater range of lipid-soluble metabolites as well as some more water-soluble metabolites that are on, as well as within, cells. Thus, methanol extraction, followed by the ethyl acetate/water partitioning, will extract a greater range of compounds and will extract compounds from within cells that would not be on seaweed surfaces. Drying this extract, mixing it with water (to attract the water-soluble metabolites) and ethyl acetate (to attract the lipid-soluble metabolites), allowing these solvents to separate (they do not mix), and retaining the ethyl acetate soluble constituents allowed us to obtain lipid-soluble compounds from both the surface and internal portions of the seaweed. This total lipid-soluble extract (from the ethyl acetate partition) can then be compared with the lipid-soluble extract from surfaces only (hexane extract), allowing an assessment of the allelopathic potency of lipid-soluble compounds from macroalgal surfaces versus the entire thallus.

For allelopathy bioassays, each lipid-soluble total or surface-only extract was resuspended in 1000 μL of methanol. 500 μL of this methanol extract mix was added to 196 g of Phytigel (Sigma–Aldrich, USA) and 9.5 mL of heated water, poured in a form over window screen, allowed to gel, and cut to obtain strips with a centered $1 \times 1 \times 0.1\text{ cm}$ gel square as described in Rasher and Hay (2010). Controls were created using the same procedure (including the addition of methanol), but no seaweed extract. In the field, the bioassays for each seaweed species were blocked by coral colony so that a control, a surface

lipid-soluble extract, and a total lipid-soluble extract strip were attached to different branches of the same coral colony using cable ties (N of coral colonies per seaweed species ranged from 10 to 19).

The blocked design by coral colony could result in a synergetic effect by having multiple treatments on the same colony; however, similar experiments conducted with several coral species, including the congeneric *P. damicornis*, demonstrated that over the 24-h duration of the experiment visible coral damage occurred only at areas of direct seaweed or extract contact, with effects not appearing beyond contact borders (Rasher and Hay 2010; Rasher et al. 2011; Andras et al. 2012). Thus, we chose the blocked design to minimize the potential variation among individuals, genotypes, and micro-environments. We noted no bleaching beyond borders of the experimental pads themselves. The effects of treatments on corals were assessed after 24 h using in situ pulse-amplitude-modulated (PAM) fluorometry as a measure of photochemical efficiency (Y , or effective quantum yield) of the corals' symbionts, with readings for healthy corals ranging $\sim 0.5\text{--}0.7$ and readings lower than 0.25 indicating bleached corals (Fitt et al. 2001; Rasher et al. 2011). Variance in PAM fluorometry readings are minimized by measuring dark-adapted samples (Fitt et al. 2001), but we wanted to conduct our assays under natural field conditions; this required daytime readings so that we could find our marked colonies and take measurements beneath the various treatment pads. To minimize the variance induced by environmental fluctuations (cloud cover, etc.) all readings were taken between 1400 and 1700 hrs and readings for different treatments were interspersed and blocked in time, thus preventing temporal variance from confounding treatment effects.

Because data did not meet parametric assumptions of normality and homogeneity of variances even after transformation, we performed a permutation-based analysis of variance (ANOVA) blocked by coral colony followed by a Tukey HSD test to assess differences in coral photochemical efficiency among treatments (control, surface-only, and total lipid-soluble extract). To evaluate the allelopathic potency among seaweed species, extract types, and the potential interactive effects between these factors (grouping variables), we used a two-way permutation-based ANOVA using the photochemical efficiency from each treatment relative to its control (relative effective quantum yield = $\frac{\text{treatment}}{\text{control}}$). This approach accounts for potential among individual variation in the corals' responses to the treatments and enables comparisons among species that were tested on different days. Contrasts were evaluated with a Tukey HSD test. All the analyses were performed using the package `lmPerm` in the software (R Core Team 2015; Wheeler 2010).

Results

Extracts from four of the five seaweeds used in our bioassays (*Am. rhodantha*, *As. taxiformis*, *C. fastigiata* and *D. bartayresiana*) suppressed the photochemical efficiency of *P. verrucosa*, with the potency of lipid-soluble surface extracts and of total lipid-soluble extracts not differing significantly within species (Fig. 1a). Neither surface nor total lipid-soluble extracts of *T. ornata* suppressed coral photochemical efficiency. Coral photochemical efficiency was little affected by the control strips and remained within the healthy coral range (Fitt et al. 2001), with effective quantum yield readings of ~0.55–0.65. Allelopathic potency varied among seaweeds, regardless of extract type, and with no interaction between species and extract type (two-way permutation-based ANOVA; Species: $p < 0.001$; Extract type: $p = 0.106$; Interaction: $p = 0.167$; Fig. 1b). Extracts from *As. taxiformis* and *C. fastigiata* were more allelopathic than those from *D. bartayresiana*, with *Am. rhodantha* being intermediate between these two groups. *Turbinaria ornata* had no effect and its extracts were significantly less potent than those of all other species tested. Extracts from *As. taxiformis* and *C. fastigata* suppressed coral photochemical efficiency by 50–70%, *Am. rhodantha* by 20–50%, and *D. bartayresiana* by 20–30%.

Discussion

Our findings suggest that the most allelopathic metabolites are either on seaweed surfaces at concentrations sufficient to saturate suppressive effects of coral photosynthesis, even if much of the compound is held internally, or deployed primarily on seaweed surfaces. Patterns for all of the allelopathic seaweeds we tested parallel a more quantitative investigation for a single allelopathic algal metabolite that was detected at active concentrations on the surface of the red alga *Phacelocarpus neurymenioides* (Andras et al. 2012). Our findings also corroborate an earlier study showing bioactive metabolites deployed on the surface of a different seaweed species, *Callophycus serratus*, with most bioactive compounds being sufficient to suppress a pathogenic fungus at surface concentrations, and the combined effects of all surface compounds certainly being adequate despite larger concentrations of active compounds also being stored within plant tissues (Lane et al. 2009).

Some bioactive secondary metabolites from seaweeds are held in specialized gland cells located within the seaweed thallus with pores allowing movement of the metabolites to seaweed surfaces (Dworjanyn et al. 1999; Paul et al. 2006; Harder et al. 2012). These metabolites may be released onto surfaces via connecting pores or via

lysis of glands in surficial tissues. The location, sites of production, and distribution among external versus internal tissues of many compounds has not been determined, but some bioactive metabolites have been well established to occur on surfaces of both seaweeds (Schmitt et al. 1995; Dworjanyn et al. 1999; Lane et al. 2009; Andras et al. 2012) and terrestrial plants (LoPresti 2015). Whether bioactive metabolites occur in seaweeds, on seaweeds, or in both locations may play a significant role in their ecological function. In some instances, the same bioactive compounds play multiple ecological roles (e.g., as both anti-herbivore and antifouling agents; Schmitt et al. 1995; Harder et al. 2012), but in other instances they have different functional roles and may need to be differentially distributed *on* versus *in* seaweed.

As an example, the herbivore-deterrent metabolites and the allelopathic metabolites in the red seaweed *Galaxaura filamentosa* must be different compounds because when this seaweed induces greater allelopathy (due to being placed in contact with a competing coral) it becomes more palatable to herbivores; both of these effects are due to alterations in its lipid-soluble chemistry (Rasher and Hay 2014). It is also of interest that, despite some 15 publications describing 27 different secondary metabolites of *Galaxaura* (MarinLit search; May 2016), none of these known bioactive metabolites were identified as being allelopathic to corals. The allelopathic metabolites may have remained undiscovered despite extensive chemical investigations due to being produced at, and significantly allelopathic at, extremely low concentrations (e.g., 0.032–0.12 $\mu\text{g g}^{-1}$ dry mass; Rasher et al. 2011).

Two allelopathic acetylated diterpenes have been isolated and identified from the green seaweed *Chlorodesmis fastigiata*. One is in the surface extracts at only 0.43–7% of its concentration in entire extracts, but it is just as active at this lesser concentration as is the entire lipid-soluble extract (Rasher et al. 2011). The other compound strongly suppressed coral photochemical efficiency at natural concentrations, but was present in such small concentrations in both the surface and entire lipid-soluble extract that its relative concentrations in each could not be determined. *Chlorodesmis* is well known as producing the cytotoxic major metabolite chlorodesmin, but this was not identified as being allelopathic in bioassays against corals (Rasher et al. 2011). Together, the findings for *Chlorodesmis* and *Galaxaura* suggest that the major, well understood natural products from seaweeds should not be assumed to be the allelopathic compounds, and that allelopathic compounds may occur both *on* and *in* seaweeds, but that surface concentrations alone appear adequate to have strong (and apparently saturating) effects on coral competitors. That low quantities of hexane-soluble surface extracts would have been as bioactive as much higher amounts of ethyl

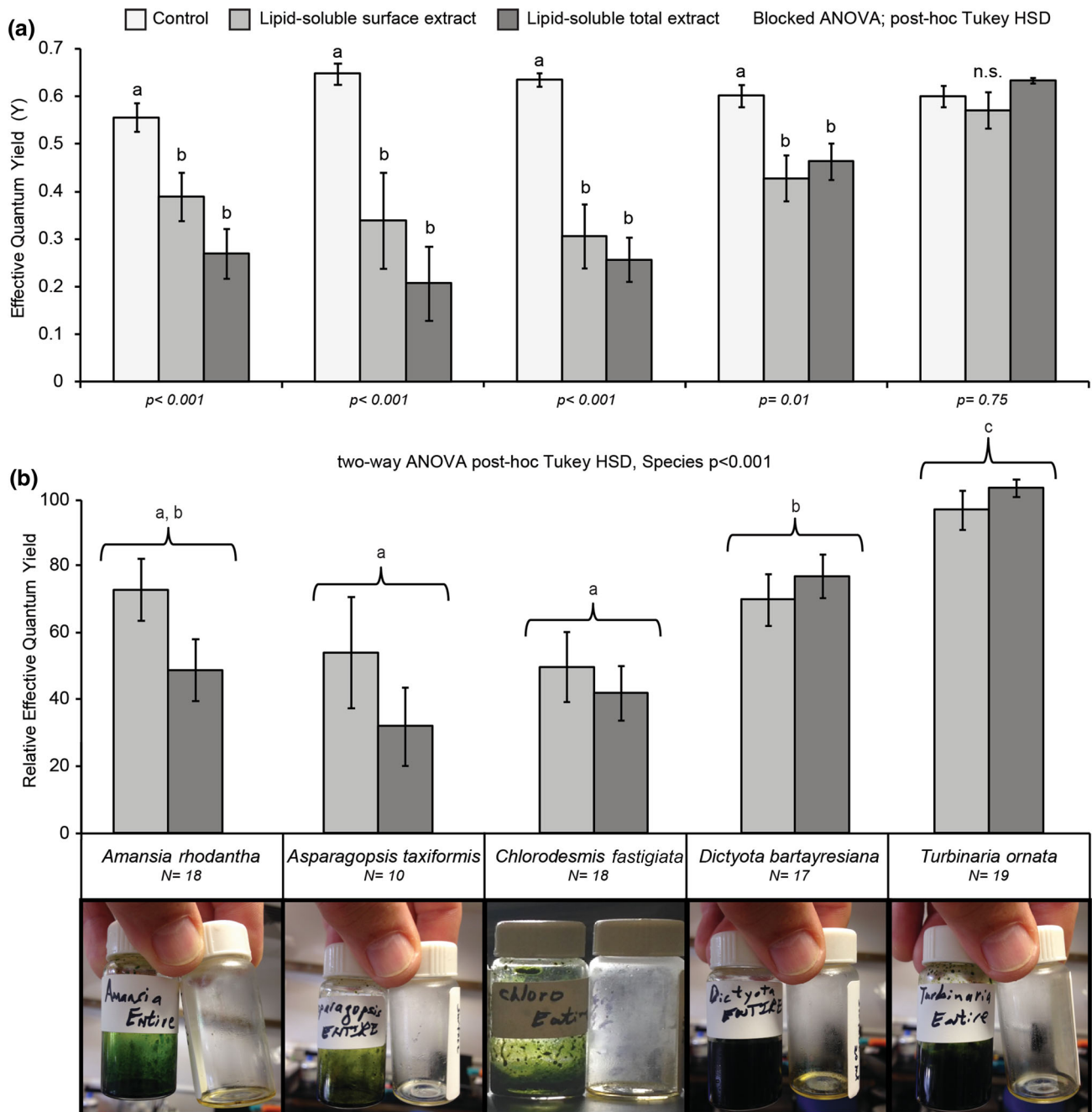


Fig. 1 Effects of seaweed surface-only and total lipid-soluble extracts on the coral *Pocillopora verucosa*. **a** Effective quantum yield (Y ; mean \pm SE) of corals exposed for 24 h to gel pads containing surface-only extracts (light gray), total lipid-soluble extracts (dark gray), and controls (gel pads with solvent, but no extracts; clear). Letters above bars indicate significant groupings

within species (p values presented below each species; *n.s.* not significant). **b** Relative effective quantum yield (Y treatment/ Y control; mean \pm SE). Letters above bars indicate post hoc comparisons within the significant factor species. Below each species is the visual comparison of vials containing total lipid-soluble extracts (left vial) and surface lipid-soluble extracts (right vial) for each seaweed species

acetate extracts from entire plants is surprising given that one would expect greater concentrations of the active compounds in the latter extract. This warrants further investigation once the specific bioactive compounds have been isolated and identified. Two of the three or more allelopathic compounds from *C. fastigiata* are known

(Rasher et al. 2011), but allopathic compounds from *Amansia*, *Asparagopsis*, and *Dictyota* have not been identified.

Not all chemically rich seaweeds deploy significant proportions of their bioactive metabolites on their surfaces. The red seaweed *L. obtusa*, for example, produces and

stores elatol within the thallus but is able to transport it to the plant surface through channel-like membranous connections (Sudatti et al. 2008). Yields of this compound from whole thalli extracts significantly reduced herbivory by sea urchins and crabs (Pereira et al. 2003), and fouling by turf-forming seaweeds and balanid crustaceans (da Gama et al. 2002). However, when similar experiments were performed using elatol at surface concentrations, neither herbivory nor biofouling were suppressed, presumably because of its low concentration on the seaweed surface (Sudatti et al. 2008). Selection due primarily to herbivory rather than biofouling/allelopathy could result in this differential allocation of bioactive metabolites to internal tissues versus external seaweed surfaces (Steinberg and de Nys 2002; Nylund et al. 2007; LoPresti 2015). As herbivores bite into and chew tissues, they will rupture cells and be exposed to whole thalli concentrations of compounds, making tests of whole extracts ecologically realistic for most herbivores, but potentially unrealistic for fouling organisms or competitors that will contact surface, but not internal, concentrations of these compounds (Pereira et al. 2003). Compounds involved in allelopathy or antifouling should be selected to be on the surfaces of organisms and in sufficient concentration to be effective against the competitors or pathogens contacting these surfaces (Steinberg and de Nys 2002; Nylund et al. 2007; Lane et al. 2009). It is possible that some allelopathic compounds are deployed primarily, or almost exclusively, on seaweed surfaces, but our findings more likely result from surface concentrations being sufficient to saturate allelopathic impacts, rather than from all of the allelopathic compounds being on the surfaces alone.

To our knowledge, this is the first study to demonstrate the allelopathic potency of lipid-soluble compounds from *Am. rhodantha* and *As. taxiformis* against corals. The allelopathic potency of extracts from *As. taxiformis* was similar to those from *C. fastigata*. In previous assays, direct contact with the seaweed *C. fastigata* was also allelopathic to the corals *Acropora aspera*, *A. millepora*, *A. nasuta*, *Montipora digitata*, *Porites cylindrica*, *P. lobata* and *Pocillopora damicornis*, suppressing their photochemical efficiency by 50–100% and eventually causing mortality (Rasher and Hay 2010; Rasher et al. 2011; Bonaldo and Hay 2014). The coral *A. nasuta* has even been selected to chemically detect contact by *Chlorodesmis* and cue mutualistic fishes to remove this seaweed (Dixson and Hay 2012).

In addition to the lipid-soluble extracts tested here, *Am. rhodantha* also may release significant amounts of water-soluble dissolved organic carbon (DOC) that can affect coral-associated microbes (Nelson et al. 2013); this effect was experienced within only μm to mm of the alga's surface (Jorissen et al. 2016). This disruption of a coral's

mutualistic microbiome is another mechanism by which seaweeds may harm corals or even cause mortality (Barott et al. 2011; Morrow et al. 2012, 2013). Microbiome disruption due to water-soluble DOC would not have occurred in our bioassays because our procedures tested only lipid-soluble extracts.

The genus *Dictyota* is allelopathic to corals both in the Caribbean (*Porites porites*) and in the Pacific (*A. millepora*, *M. digitata*, *P. cylindrica*; Rasher and Hay 2010; Rasher et al. 2011) where they are among the most common seaweeds contacting corals and are frequently associated with disturbed reefs (Barott et al. 2011; Morrow et al. 2013; Bonaldo and Hay 2014; Longo and Hay 2015). Extracts of two species within this genus (*D. pinnatifida* and *D. pulchella*) negatively affected larval settlement, recruitment, and survival of both larvae and new recruits of the coral *P. astreoides* in the Caribbean (Paul et al. 2011). Thus, the prevalence of *Dictyota* in seaweed-dominated reefs may go beyond direct competition with adult corals (the allelopathy we investigated here); they may also suppress larval settlement and thus reef recovery following coral losses.

Turbinaria ornata can be abundant at degraded reefs in the Pacific (Rasher et al. 2013) and its abundance has increased in French Polynesia since the 1980s (Bittick et al. 2010). This species competes with corals via shading and abrasion, but it was not allelopathic in our assays, nor in earlier assays against *A. millepora*, *M. digitata*, or *P. cylindrica* (Rasher and Hay 2010; Rasher et al. 2011). Thus, the tolerance, or susceptibility, of the coral *Pocillopora verrucosa* to allelopathy of common seaweeds seems comparable to other branching species in the Pacific. These findings could be particularly important for the reefs in Moorea that are recovering from crown-of-thorns outbreaks and cyclone disturbances (Adjeroud et al. 2009; Traçon et al. 2011), and where several reefs are dominated by *Pocillopora* (Edmunds 2012).

For the allelopathic species we investigated, lipid-soluble surface extracts were as potent as entire lipid-soluble extracts, suggesting that these compounds were deployed externally in sufficient concentration to be effective in surface-mediated interactions such as allelopathy. Thus, numerous seaweeds may act as toxic paint brushes, affecting corals on contact, but not at a distance. This may be a strategy to preserve bioactive metabolites nearby where they can advantage the producer rather than releasing them to currents where compounds would be rapidly advected away.

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