



## Palatability and chemical defences of benthic cyanobacteria to a suite of herbivores



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### ABSTRACT

Nuisance blooms of toxic cyanobacteria are a common occurrence in many tropical and subtropical locations. Benthic marine cyanobacteria of the genera *Lyngbya*, *Okeania*, and *Moorea* are frequently observed in both Florida and throughout the Caribbean, sometimes forming large mats, and are prolific producers of bioactive secondary metabolites that often act as feeding deterrents to generalist herbivores. Little is known regarding the ecological roles of the secondary metabolite chemistry and the palatability of benthic cyanobacteria to grazers. This study examines the palatability of benthic cyanobacterial species from Florida (IRL1, IRL2, IRL3 and *Okeania erythroflocculosa*) and Belize (BEL1, BEL2) to a range of macro- and mesograzers in Florida and Belize. Pair-wise feeding assays using artificial diets of *Gracilaria tikvahiae* or fish food coated with cyanobacterial extracts and a control were used to determine palatability of extracts to Floridian and Belizean generalist grazers. The extracts of IRL1, IRL2, IRL3 and *O. erythroflocculosa* from Florida did not deter feeding by invertebrate grazers. Reef fish, however, were deterred by the non-polar extracts of IRL1, IRL3 and *O. erythroflocculosa*. *Stylocheilus striatus* was stimulated to feed on IRL2 extracts and non-polar extracts from IRL3. Non-polar extracts of BEL1 stimulated feeding in *S. striatus*; however, no significant difference was observed between BEL2 extracts and the control. Most generalist invertebrate grazers, sympatric and non-sympatric, appear indifferent to cyanobacteria extracts whilst reef fish are more likely to be deterred by cyanobacterial extracts, which may affect species interaction within communities with fluctuating or dominating benthic cyanobacterial blooms.

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### 1. Introduction

Cyanobacteria are a ubiquitous component of all aquatic ecosystems. The family Oscillatoriaceae consists of more than 800 species with many species producing secondary metabolites that are of interest both ecologically and pharmaceutically (Engene et al., 2013a). One genus that sparked a wealth of research in these areas over the past five decades is *Lyngbya*. However, recent taxonomic reclassification of this polyphyletic group has yielded several phylogenetically distinct lineages including *Okeania* and *Moorea* (Engene et al., 2012; Engene et al., 2013a, 2013b). *Lyngbya*, *Okeania* and *Moorea* are abundant and globally

distributed genera in tropical and subtropical marine benthic environments (Engene et al., 2012; Engene et al., 2013a, 2013b).

Cyanobacterial blooms appear to be increasing in frequency and severity at a number of locations around the world (Albert et al., 2005; Paerl and Huisman, 2009; Paerl and Paul, 2012; Paul et al., 2005) and as such, they can have significant economic impacts upon recreational activities and fisheries (Abal et al., 2001). Benthic cyanobacteria are commonly found in Florida (Arthur et al., 2009; Burns, 2008; Engene et al., 2013b; Paul et al., 2005; Sharp et al., 2009) and have been observed in Belize on a number of occasions (Gunasekera et al., 2008a; McClanahan et al., 2007). Blooms can be prevalent during the summer and fall and are often exacerbated by warm water temperatures (>24°C) in relatively calm, shallow waters (Watkinson et al., 2005; pers. obs.). The Oscillatoriaceae family of benthic cyanobacteria have great plasticity allowing them to survive and thrive on a wide variety of substrates including sand, seagrass, algae, mangrove roots and coral reefs (Engene et al., 2013b; Paul et al., 2005).

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Species of *Lyngbya*, *Moorea* and *Okeania* are prolific secondary metabolite producers (Blunt et al., 2010; Blunt et al., 2012; Engene et al., 2012, 2013a; Liu and Rein, 2010). Why such huge arrays of compounds are produced and why they often vary geographically is unknown, but genetics (Sharp et al., 2009), local physico-chemical conditions (Paerl, 1996), nutrient availability (Arthur et al., 2009) and grazer interactions (Hay and Fenical, 1988; Paul et al., 2001) have all been suggested as driving factors. Many of these secondary metabolites act as feeding deterrents to a range of generalist grazers such as crabs (Pennings et al., 1996), sea urchins (Capper et al., 2006a; Nagle et al., 1996) and fish (Capper et al., 2006b; Nagle and Paul, 1998); often allowing blooms to proliferate when environmental conditions are favourable (Paerl and Paul, 2012; Paul et al., 2007; Thacker and Paul, 2001).

Given the vast array of cyanobacterial compounds that can vary both temporally and spatially, it is very difficult to compare palatability responses for generalist herbivorous grazers. Sensitivity to compounds and palatability can vary between grazers (Capper et al., 2006a), and prior exposure to 'local' compounds may acclimate local grazers but deter grazers in other geographic locations. Capper et al. (2006a) hypothesised that *Lyngbya* compounds are likely to be locally rather than broadly deterrent across a geographical range. However, this was not found to be the case when a crude extract was tested against sympatric and allopatric grazers in two geographic locations, Guam and Australia. Deterrence was observed in most consumers regardless of geographic origin or prior exposure to 'local' compounds. The major compound isolated during this study was the highly toxic lyngbyatoxin A (LTA). It is not known whether benthic cyanobacteria with less 'potent' toxins would be equally deterrent to grazers in different geographical locations. Thus far, collections of benthic cyanobacteria from Florida and Belize have not yielded LTA (Engene et al., 2013a, 2013b; Kwan et al., 2010; Sharp et al., 2009).

While benthic cyanobacteria blooms often play host to a range of macro- and mesograzers (Cruz-Rivera and Paul, 2002), the majority of these grazers appear to be opportunistic rather than specialists (Cruz-Rivera and Paul, 2002, 2006; Watkinson et al., 2005). Even for specialist grazers, sensitivity to crude extracts or specific compounds can vary both within and among species of cyanobacteria. The 'specialist' grazer *Stylocheilus striatus*, an opisthobranch mollusc, can be stimulated to feed in the presence of specific compounds (Arthur et al., 2009; Capper et al., 2005; Nagle et al., 1998). Nagle et al. (1998) found that malylgamides and majusculamides increased feeding at lower concentrations, but inhibited feeding at higher concentrations. While *S. striatus* may be able to bioaccumulate *Lyngbya* compounds with no apparent detrimental impact (Capper et al., 2005; Pennings and Paul, 1993a), sensitivity to, and thus avoidance of different species of benthic cyanobacteria may ultimately increase animal fitness. In palatability and associated biomass increase assays, *S. striatus* showed a preference for *L. polychroa* (now known as *Okeania erythroflocculosa*, Engene et al., 2013b) over *L. cf. confervoides* and attained a greater biomass on a monospecific diet of *L. polychroa* (Capper and Paul, 2008).

To further investigate deterrence of cyanobacteria to generalist and specialist consumers, the palatability of a range of benthic cyanobacteria and an unknown consortium of cyanobacterial species in sub-tropical Florida and tropical Belize was examined. Feeding experiments with a range of cyanobacterial extracts were conducted to test the adaptability and tolerance of diverse consumers that feed on chemically defended prey.

## 2. Materials and methods

### 2.1. Study sites and organisms

Grazers and cyanobacteria were collected and tested in Belize or Florida; however, it is important to note that both are broadly

distributed across the Florida and Caribbean region (Engene et al., 2012, 2013a, 2013b; Paul et al., 2005). Repeated collections of cyanobacteria from the same locations in both these regions over the years means they can readily be identified based on morphology and secondary metabolites, which are characteristic for each species (Engene et al., 2013a and b Sharp et al., 2009). Choosing these two locations therefore, allowed an assessment of a broad array of cyanobacteria-grazer interactions.

#### 2.1.1. Florida collections

Blooms of cyanobacteria were observed at two sites in the Indian River Lagoon (IRL) at Jensen Beach in St. Lucie County during August 2004 with a different morphology collected at each location: 'filamentous' (IRL1) and 'slimy' (IRL2) (Table 1). Although previously both morphotypes were labelled as *Lyngbya cf. majuscula* (Dobretsov et al., 2010; Kwan et al., 2011), a recent phylogenetic study has now classified them as distinct from *Lyngbya* spp. based on phylogenetic analysis of 16SrRNA genes (Clade IV in Engene et al., 2013a). Other benthic cyanobacteria can be less conspicuous in the IRL; however, a fortuitous finding of one cyanobacterial mat consortium (covering approx. 3 m<sup>2</sup>) attached to a *Cladophora* species was collected in November 2004 (IRL3) at a small island located to the south of the Smithsonian Marine Station at Fort Pierce (SMSFP). Unfortunately, the composition of this particular cyanobacterial mat was not confirmed. *Okeania erythroflocculosa* (previously known as *Lyngbya polychroa* (red), Sharp et al., 2009) was collected in Broward County, Florida and maintained as above. All collections were returned to the SMSFP and maintained in 5 gal buckets of seawater at 35 ppt at 24°C with aeration and a 12 h light and dark cycle. Water was changed every 24 h. *Gracilaria tikvahiae* was obtained from Harbor Branch Oceanographic Institute and freeze-dried for use in palatability assays to test the effect of chemical extracts on feeding.

Sea hares (*S. striatus*) were collected from the blooms of *O. erythroflocculosa* and *Lyngbya cf. confervoides* in Broward County and maintained in small aquaria (1 L) at SMSFP on a diet of their host algae. Sea urchins (*Echinometra lucunter*) and crabs (*Pachygrapsus transversus*) were collected from the rocky shores adjacent to SMSFP and maintained in 5 gal buckets. Salinities were kept between 34 and 36 ppt and temperature consistent with ambient (24°C) with 12 h light and dark cycles. Water was changed every 24 h.

#### 2.1.2. Belize collections

Two benthic cyanobacteria were collected during the spring of 2005. The first was identified as BEL1, red in colour (formerly *Lyngbya cf. polychroa*, but now called *Moorea producens*, Engene et al., 2012) and found in large quantities on mangrove roots at Twin Cays, Belize. Filament width was 27.78 μm ± 0.83 μm SE (n = 10) with a cell width of 25.28 μm ± 0.83 μm SE and length of 15 μm ± 0.00. The second was green in colour, BEL2 (formerly *Lyngbya cf. majuscula*, BEL2) and was collected from mangrove roots in the Pelican Cays. Filament width was 27.44 μm ± 0.64 μm SE (n = 10) with a cell width of 22.44 μm ± 0.64 μm SE and length of 5.0 μm ± 0.00. Collections were returned to the Smithsonian laboratory at Carrie Bow Cay (CBC) and maintained in 5 gal buckets of seawater at 35 ppt at ambient temperature (27°C) with aeration with 12 h light and dark cycles.

Sea urchins (*Diadema antillarum*) and crabs (*Pitho aculeata* and *Mithraculus sculptus*) were collected locally on the reef surrounding CBC. The sea hare *S. striatus* was not observed in any cyanobacterial collections in Belize and therefore could not be utilized in assays at CBC. Sea urchins were maintained in 5 gal buckets with flow-through seawater. Mesograzers were maintained in individual plastic containers with seawater changed every 24 h. All animals were kept at ambient temperatures with 12 h light and dark cycles. In-situ fish assays were carried out on Golden Reef close to CBC.

**Table 1**  
Geographic location of collection sites for cyanobacteria and cyanobacteria consortia in Florida and Belize and herbivorous species used in palatability assays.

Cyanobacteria	Collection site	Collection site co-ordinates	Collection date	Substrate	Crude extract yield (%) <sup>c</sup>	Assay location <sup>a</sup>	Herbivores used in assays
IRL1 ('filamentous')	Indian River Lagoon (IRL), Jensen Beach, Florida	27° 13' 674" N 80° 12' 668" E	August 2004	Seagrass <i>Thalassia testudinum</i>	NP = 6.72 P = 6.03	CBC CBC CBC GR SMS SMS	Cephalaspidean <sup>b</sup> Sea urchin ( <i>Diadema antillarum</i> ) Majid crab ( <i>Pitho aculeata</i> ) Reef fish (mixed assemblage) Sea urchin ( <i>Echinometra lucunter</i> ) Grapsid crab ( <i>Pachygrapsus transversus</i> )
IRL2 ('slimy')	Indian River Lagoon (IRL) Jensen Beach, Florida	27° 14' 563" N 80° 13' 376" E	August 2004	Bare sediment	NP = 2.07 P = 5.21	CBC SMS SMS	Sea urchin ( <i>Diadema antillarum</i> ) Sea urchin ( <i>Echinometra lucunter</i> ) Sea hare ( <i>Stylocheilus striatus</i> )
Cyanobacterial mat mixed consortia IRL3	Small island located south of SMS, Fort Pierce, Florida	27° 26' 716"N 80° 18' 698" W	November 2004	<i>Cladophora</i> sp.	NP = 11.53 P = 6.07	CBC CBC CBC GR SMS SMS SMS	Sea urchin ( <i>Diadema antillarum</i> ) Majid crab ( <i>Pitho aculeata</i> ) Emerald crab ( <i>Mithraculus sculptus</i> ) Reef fish (mixed assemblage) Sea urchin ( <i>Echinometra lucunter</i> ) Grapsid crab ( <i>Pachygrapsus transversus</i> ) Sea hare ( <i>Stylocheilus striatus</i> )
<i>Okeania erythroflocculosa</i>	Off Fort Lauderdale, Broward Co. USA	26° 15' 1344"N 80° 03' 9077"W	May - July 2005	Soft corals	NP = 11.74 P = 18.39	CBC CBC	Sea urchin ( <i>Diadema antillarum</i> ) Reef fish (mixed assemblage)
Cyanobacteria BEL1 (red)	Twin Cays, Belize	16° 08' 158"N 088° 00' 991"W	March 2005	Mangrove roots	NP = 25.65 P = 20.97	SMS	Sea hare ( <i>Stylocheilus striatus</i> )
Cyanobacteria BEL2 (green)	Pelican Cays, Belize	16° 6' 487" N 088° 01' 990"W	March 2005	Mangrove roots	NP = 33.50 P = 33.80	SMS	Sea hare ( <i>Stylocheilus striatus</i> )

<sup>a</sup> CBC refers to Carrie Bow Cay Marine Station, Belize; GR refers to Golden Reef, Belize; SMS refers to Smithsonian Marine Station, Fort Pierce, Florida, USA.

<sup>b</sup> Unidentified cephalaspidean species (confirmed by Dr. Paula Mikkelsen).

<sup>c</sup> NP = non polar, P = polar % of dry weight.

### 2.1.3. Chemical extraction of cyanobacteria

To obtain crude extracts for use in palatability assays, all cyanobacteria were frozen at -20°C and then freeze-dried. Dried material was ground, then extracted four times during a 48 h period in ethyl acetate:methanol (1:1) for the non-polar extract; then three times during a 36 h period in ethanol:distilled water (1:1) for the polar extract. Samples were filtered and dried by rotary evaporation. Presence of secondary metabolites in crude extracts was confirmed using proton Nuclear Magnetic Resonance (NMR) spectroscopy.

### 2.1.4. Palatability assays using cyanobacterial extracts

To determine whether secondary metabolites deter feeding on cyanobacterial species, both non-polar and polar extracts were incorporated into artificial diets and offered to a range of meso- and macrograzers. Artificial diets containing or lacking cyanobacterial crude extracts were created using methods outlined in Hay et al. (1998). Non-polar extracts were resolubilized in ethyl acetate and methanol (1:1) and polar extracts in methanol. Both extracts were then coated at natural concentration by percentage dry mass onto freeze-dried, powdered *Gracilaria tikvahiae*. This natural concentration was based on the quantity of crude extract obtained from a known quantity of freeze-dried cyanobacteria. It should be noted that yields from IRL2 were much less than other cyanobacteria extracted, and as such, were not used in all palatability assays. This extraction method has been successfully used to test extracts and compounds from other marine cyanobacteria (Capper et al., 2006c; Cruz-Rivera and Paul, 2007; Nagle and Paul, 1998). *G. tikvahiae* was chosen because it is a broadly palatable alga. In previous palatability trials *G. tikvahiae* was significantly preferred by *S. striatus* to *Ulva* spp. (Capper and Paul, 2008), and as such has been used in all subsequent assays. A rotary evaporator was used to remove all organic solvent allowing the hydrophobic and hydrophilic components of the crude extracts to adhere to the surface of *G. tikvahiae*. Control, non-polar and polar treatment foods were incorporated into agar-based artificial diets as described by Hay et al. (1998). The mixture was then poured onto plastic screen mesh (~2 mm<sup>2</sup> per square) to make

food strips (10 × 15 squares). The seahare *S. striatus* was not offered IRL1 during these feeding assays as palatability for this crude extract had already been determined (Capper and Paul, 2008).

To assess palatability of Floridian benthic cyanobacteria IRL1 and IRL2, *O. erythroflocculosa* and the cyanobacteria mat IRL3 to grazers, non-polar and polar treatment food strips plus a control were offered simultaneously to individual consumers in a choice experiment. The same assays were also utilized to assess palatability of Belizean extracts BEL1 and BEL2 to *S. striatus*. A fixed-consumption stopping rule was applied (Lockwood, 1998) where all tests were terminated once >50% of total food had been consumed. Consumption was quantified as the number of mesh squares cleared of food over time. Replicates where <10% or >90% of total food had been consumed were eliminated from each test. Data were analysed using Friedman's ANOVA and Tukey's *post-hoc* test with Sigmatat® software ( $\alpha < 0.05$ ).

Floridian *Pachygrapsus transversus* palatability assays utilized non-polar extracts of IRL1 and IRL3 only as no further polar extracts were available. A paired t-test was used to analyse data.

Reef fish feeding assays were conducted in situ (depth of 7 m) at Golden Reef, Belize to assess palatability of extracts of Floridian cyanobacterial IRL1, IRL3 and *O. erythroflocculosa*. Extracts were resolubilized and mixed with the food as above at natural concentration by percentage dry mass fish food (Kent Platinum Reef Herbivore):

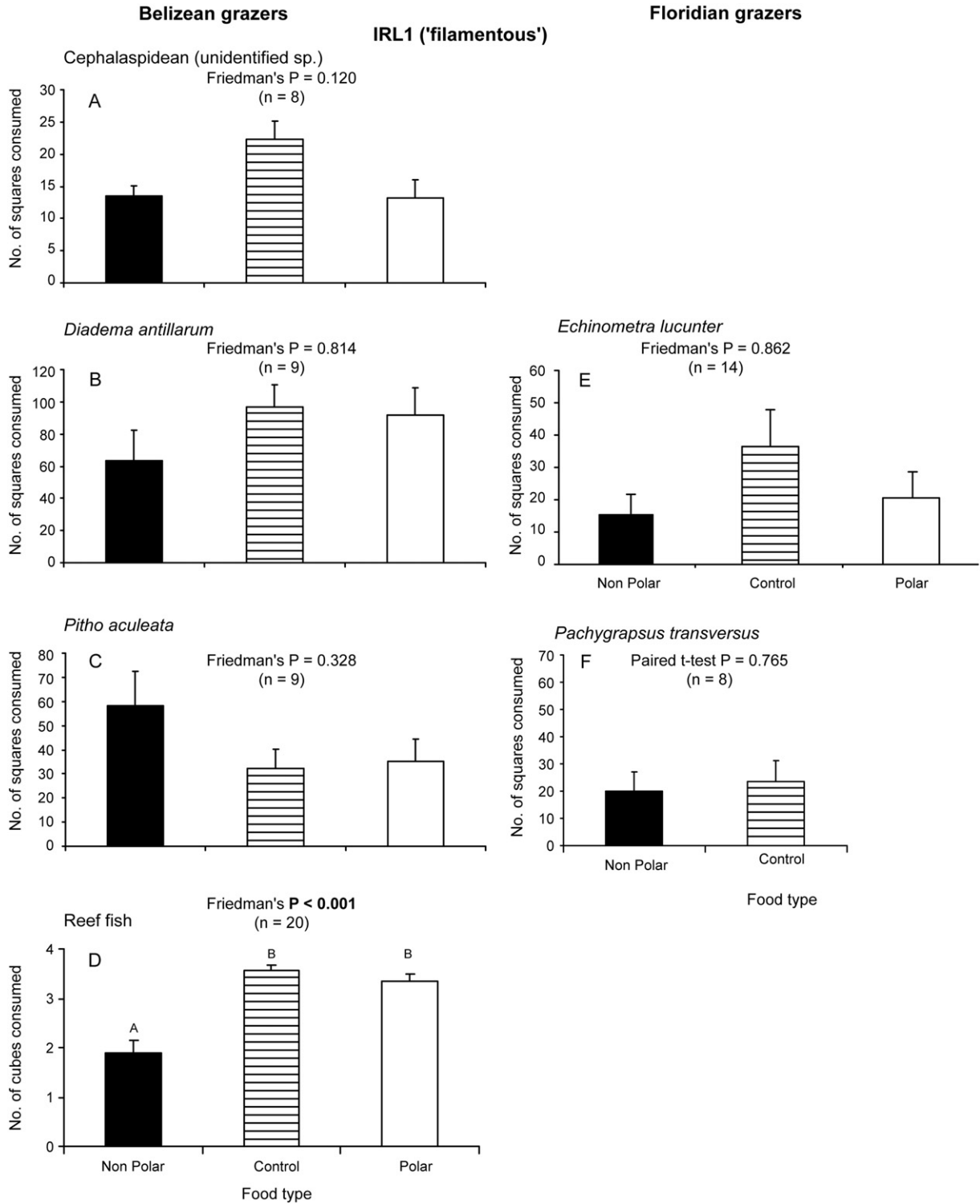
$$\text{Amount of extract added (g)} = \frac{(f + a + c)xy}{(1-y)} \quad (1)$$

Where *food weight*,  $f = 5$  g; *agar weight*,  $a = 1.25$  g; *carrageenan weight*,  $c = 1.25$  g and  $y = \text{extract weight (g)}/\text{initial dry weight of cyanobacterium before extraction}$ .

Ingredients were mixed with 100 ml of rainwater (collected in cisterns) and the mixture was heated to boiling in a microwave. Cyanobacterial extracts were added to the mixture and poured into a mould to make food cubes (1 cm<sup>3</sup>). These were then attached to polypropylene ropes as per Pennings et al. (1999). The same volume of solvent only was added to control foods. Ropes were assigned with

either non-polar, polar or control cubes and placed on the reef where an assemblage of reef fish was able to choose among the ropes. Each rope contained four cubes per replicate, with twenty ropes of each treatment type used per experiment. Sets of three ropes (control, non-polar and polar extracts) were placed on the reef and were scored

when >50% of total food had been consumed. Data were analysed using Friedman's ANOVA and Tukey's *post-hoc* test with Sigmastat® software ( $\alpha < 0.05$ ). Fish feeding assays using Floridian *L. majuscula* (IRL2) were not possible as only a small quantity of the crude extract was available.



**Fig. 1.** Palatability assay for Indian River Lagoon IRL1 crude extracts using Belizean grazers: (A) cephalaspidean (unidentified sp.) (B) sea urchin *Diadema antillarum*; (C) crab *Pitho aculeata* and (D) reef fish; and Floridian grazers: (E) sea urchin *Echinometra lucunter*; and (F) crab *Pachygrapsus transversus*. Assays used an artificial diet of solvent treated *Gracilaria tikvahiae* (☐), control; or *G. tikvahiae* with non-polar (■) or polar (□) crude extracts of IRL1 treatments. Data are mean squares/cubes consumed per animal ( $\pm$  SE). Friedman's ANOVA was used to analyse data (A)–(E). A paired t-test was used to analyse data (F). Letters above bars denote a significant difference using a non-parametric Tukey's *post-hoc* analysis ( $P < 0.05$ ) ( $n$  = number of replicates). N.B. Scales on axes vary between graphs.

### 3. Results

#### 3.1. Palatability assays using cyanobacterial extracts

##### 3.1.1. Belize assays

Belizean invertebrates were not deterred or stimulated to feed by Floridian cyanobacterial extracts. IRL1 did not deter feeding in cephalaspideans (Friedman's ANOVA,  $P = 0.120$ , Fig. 1A). Sea urchins *Diadema antillarum* were equally undeterred by IRL1, IRL2, IRL3 and *Okeania erythroflocculosa* extracts (Friedman's ANOVA,  $P = 0.814$ , Fig. 1B;  $P = 0.285$ , Fig. 2A,  $P = 0.258$ , Fig. 3A and  $P = 0.118$ , Fig. 4A). Whilst Floridian IRL3 polar extracts appeared to deter feeding in majid crabs *P. aculeata* (Friedman's ANOVA,  $P = 0.050$ , Fig. 3B), *post-hoc* analysis failed to identify significant differences. These crabs were also indifferent to IRL1 (Friedman's ANOVA,  $P = 0.328$ , Fig. 1C), with green crabs *Mithraculus sculptus* showing no preference for IRL3 extracts (Friedman's ANOVA,  $P = 0.172$ , Fig. 3C).

Reef fish were deterred by non-polar IRL1 (Friedman's ANOVA,  $P < 0.001$ , Fig. 1D), non-polar cyanobacteria mat consortia IRL3 (Friedman's ANOVA,  $P < 0.001$ , Fig. 3D) and non-polar extracts of *O. erythroflocculosa* (Friedman's ANOVA,  $P < 0.022$ , Fig. 4B).

##### 3.1.2. Florida assays

Floridian sea urchins, *E. lucunter*, were not deterred by IRL1, IRL2 or IRL3 extracts (Friedman's ANOVA,  $P = 0.862$ , Fig. 1E;  $P = 0.182$ , Fig. 2B;  $P = 0.336$ , Fig. 3E). The Floridian crab *P. transversus* was also undeterred by IRL1 and IRL3 non-polar extracts (paired *t*-test,  $P = 0.765$ , Fig. 1F;  $P = 0.750$ , Fig. 3F).

The specialist grazer *S. striatus* was stimulated to feed on both Floridian IRL2 non-polar and polar extracts (Friedman's ANOVA,  $P = 0.047$ , Fig. 5A) and non-polar extracts from IRL3 (Friedman's ANOVA,  $P = 0.003$ , Fig. 5B). Non-polar extracts of Belizean cyanobacteria BEL1 also stimulated feeding in *S. striatus* (Friedman's ANOVA,  $P = 0.002$ , Fig. 5C), whereas no significant difference was observed between BEL2 extracts and the control (Friedman's ANOVA,  $P = 0.242$ , Fig. 5D).

### 4. Discussion

The complex interactions that exist between herbivorous grazers and cyanobacteria provide us with an opportunity to understand the ecological role secondary metabolites may play in marine environments. Understanding the chemistry and how it affects consumer grazing patterns is essential to help understand the potential of these blooms to persist. The focus of this study was to determine the responses of a suite of herbivores to different cyanobacterial extracts

and explore the potential role of those compounds. Generalist invertebrate grazers were indifferent to cyanobacterial extracts (regardless of geographical location and prior exposure). This is an interesting finding as many *L. majuscula* compounds have previously demonstrated negative feeding cues to a suite of generalist herbivores (Cruz-Rivera and Paul, 2007; Nagle and Paul, 1999). Reef fish were deterred by extracts from IRL1, IRL3 and *O. erythroflocculosa*. *S. striatus* were stimulated to feed by three out of the four cyanobacteria extracts, reiterating their role as a specialist grazer of cyanobacteria.

Many studies have reached the conclusion that *Lyngbya* and related benthic marine cyanobacteria are unpalatable to generalist grazers due to the presence of secondary metabolites (Capper et al., 2006b, 2006c; Cruz-Rivera and Paul, 2007; Nagle et al., 1996; Nagle and Paul, 1998, 1999; Pennings et al., 1996). Blooms also differ greatly in their 'signature chemistry' which is primarily dependent upon genetics (Engene et al., 2013a; Engene et al., 2013b; Sharp et al., 2009), and may make them unique to a particular location. It is predicted that 'local' generalist grazers experience negative feeding cues due to frequent exposure, thus allowing these nuisance blooms to propagate and proliferate. But local grazers may become tolerant to secondary metabolites encountered as repeated exposure to crude extract may increase feeding if no deleterious effects ensue. Capper et al. (2006a) found invertebrate generalist grazers were deterred by the conglomerate of chemicals found in *L. majuscula* regardless of prior contact or association with *Lyngbya* blooms or geographical location. It is likely that this deterrence was related to secondary metabolite composition. In the current study, comparisons between sympatric and allopatric grazers could not be made due to availability of different herbivores in each location and the logistics of running all assays with the same grazers.

In this study, fish appeared to be more discerning or sensitive to secondary metabolites than other generalist grazers. Belizean reef fish were deterred by extracts of IRL1, IRL3 and *O. erythroflocculosa*. Capper et al. (2006c) also showed that rabbitfish (*Siganus fuscescens*) would reject *L. majuscula* containing lyngbyatoxin-A (LTA) and choose to starve rather than consume the chemically defended cyanobacterium. However, when LTA was not detectable fish did consume *L. majuscula* voraciously (Capper, unpubl. data). A mass die-off of rabbitfish in Guam was attributed to fish starving to death even though *L. majuscula* was the most abundant food source in the area (Nagle and Paul, 1998). Ypaoamide was isolated from these blooms and deterred feeding in rabbitfish in the laboratory (Nagle et al., 1996). The presence of secondary metabolites appears to be essential for survival of cyanobacterial blooms in complex grazer community structure. These compounds allow blooms to proliferate, even in the presence of vast numbers of herbivorous grazers, which often results

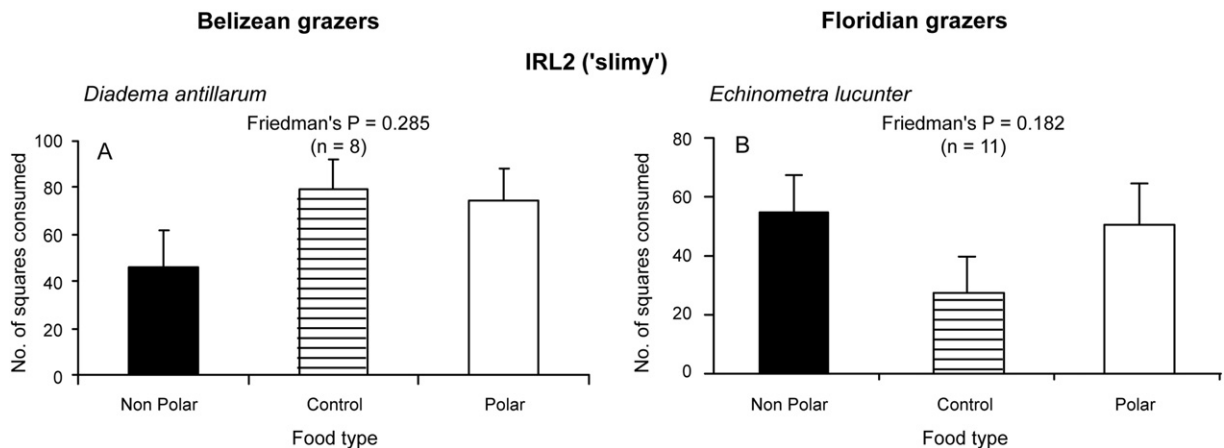
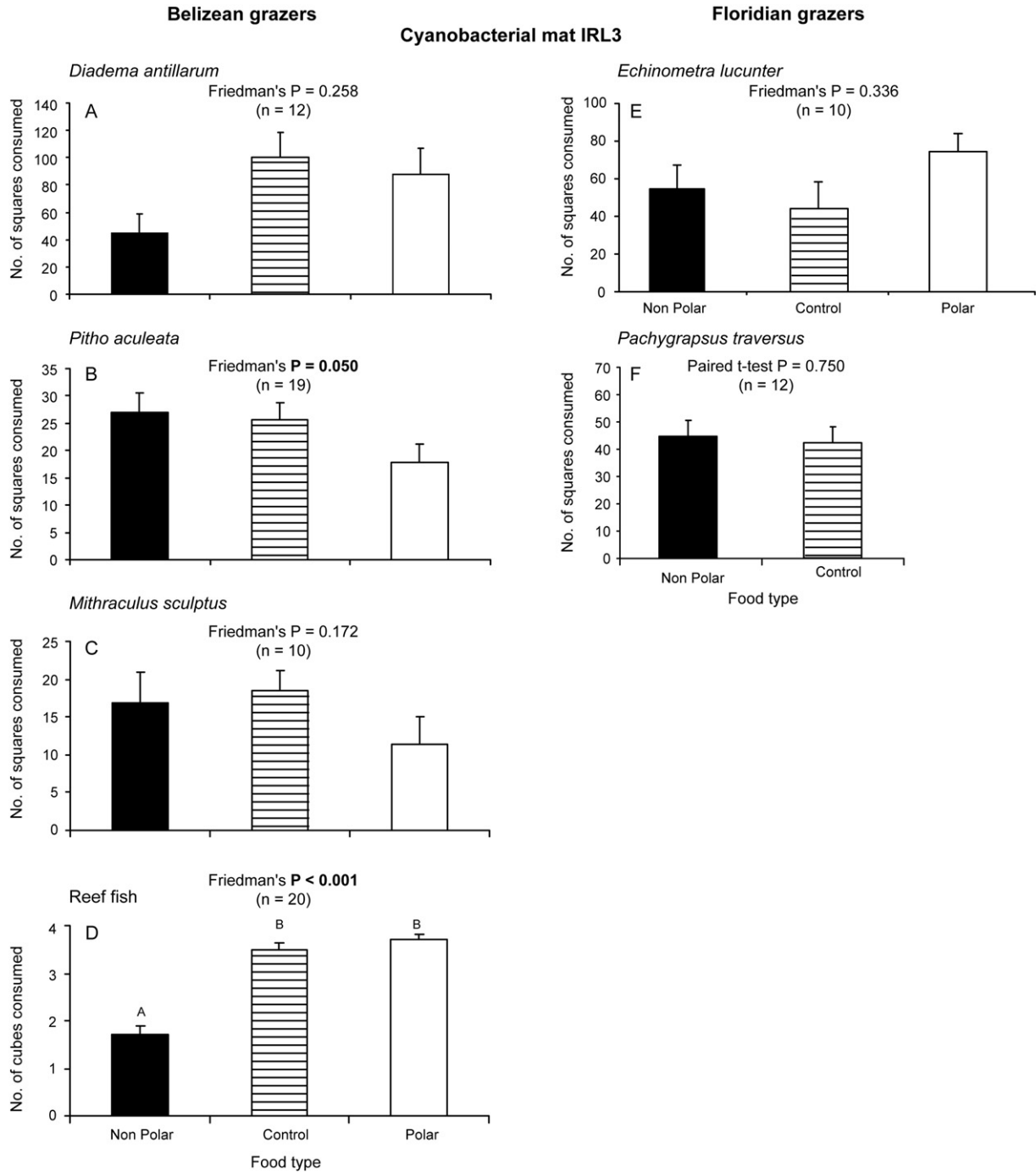


Fig. 2. Palatability assay for Indian River Lagoon IRL2 crude extracts using: (A) Belizean sea urchin, *Diadema antillarum* and (B) Floridaian sea urchin, *Echinometra lucunter*, with an artificial diet of solvent treated *Gracilaria tikvahiae* (▨), control; or *G. tikvahiae* with non-polar (■) or polar (□) crude extracts of IRL2 treatments. Data are mean squares consumed per animal (± SE). Friedman's ANOVA was used to analyse data. ( $n$  = number of replicates). N.B. Scales on axes vary between graphs.

in negative ecological and economic impacts (Abal et al., 2001; Dennison et al., 1999; Paul et al., 2001).

Whilst most algal and cyanobacterial chemical defences probably evolved in response to diffuse herbivory from diverse types of herbivores (Craft et al., 2013; Hay and Fenical, 1988), specialized herbivores such as opisthobranchs have adapted to feed on this ephemeral food source. Sea hares and cephalaspideans often feed exclusively on cyanobacteria suggesting sub- and tropical mesograzers may have evolved to consume primarily benthic cyanobacteria instead of algae (Cruz-Rivera and Paul, 2006). However, these organisms show different

degrees of trophic specialization (Cruz-Rivera and Paul, 2006). Capper and Paul (2008) observed that cephalaspideans and opisthobranch molluscs may forgo their host cyanobacteria and switch to a more palatable species should one become available. Positive chemical feeding cues or attractants are likely to be the drivers behind this host-switching phenomenon (Capper and Paul, 2008). It is very common for secondary metabolite concentrations in benthic cyanobacteria to vary between geographic locations and even within blooms in the same location (Capper et al., 2006b; Nagle and Paul, 1999). The opisthobranch's ability to detect changes in secondary metabolite concentrations of



**Fig. 3.** Palatability assay for Indian River Lagoon cyanobacteria mat IRL3 crude extracts using Belizean grazers: (A) sea urchin *Diadema antillarum*; (B) crab *Pitho aculeata*; (C) crab *Mithraculus sculptus*; (D) reef fish; and Floridian grazers, (E) sea urchin *Echinometra lucunter*; and (F) crab *Pachygrapsus transversus*. Assays used an artificial diet of solvent treated *Gracilaria tikvahiae* (□), control; or *G. tikvahiae* with non-polar (■) or polar (□) crude extracts of IRL3 treatments. Data are mean squares/cubes consumed per animal (± SE). Friedman's ANOVA was used to analyse data (A)–(E). A paired t-test was used to analyse data (F). Letters above bars denote a significant difference using a non-parametric Tukey's *post-hoc* analysis ( $P < 0.05$ ) ( $n$  = number of replicates). N.B. Scales on axes vary between graphs.

cyanobacterial hosts could decrease potential negative post-ingestive consequences and ultimately increase animal fitness (Capper and Paul, 2008; Pennings and Carefoot, 1995; Rogers et al., 1995; Thacker et al., 1997). If *S. striatus* is so suitably adapted to consume most cyanobacteria, one might assume that all cyanobacterial consortia would stimulate feeding. However, some secondary metabolites, when isolated, can cause feeding deterrence in *S. striatus*, especially at high concentrations (Nagle et al., 1998; Pennings and Paul, 1993b). In this study, while extracts of IRL2 and IRL3 did stimulate *S. striatus* to feed, IRL1 in a previous study did not (Capper and Paul, 2008). What then prompted the difference in palatability between the two related cyanobacteria? Changes in nutrient availability (e.g. P, Fe) can affect secondary metabolite concentrations in *Lyngbya* and can lead to quantifiable changes in the feeding behaviour of *S. striatus* (Arthur et al., 2009). Whilst the nutritional value of a food can be an important driver of consumption, Capper and Paul (2008) showed that food choices were not always clearly aligned with nutritional value and were more likely due to chemical composition. A previously unrecognized phylogenetic diversity of benthic cyanobacteria has recently been described (Engene et al., 2013a), so it is not surprising that chemical analysis of these two strains revealed differing chemical composition with the major secondary metabolite in IRL1 being lyngbyoic acid (Kwan et al., 2010), whilst IRL2 contained malyngolide (Dobretsov et al., 2010). This suggests that it was likely the chemical composition driving *S. striatus* feeding preferences. However, Nagle et al. (1998) observed indifference in the same opisthobranchs when pure malyngolide was offered at low concentrations and *S. striatus* were actually deterred, rather than stimulated, at 'natural' concentrations in Guam. The difference between the two allopatric groups may be due to concentration differences in the crude extract depending on location, or acquired local tolerances.

Many cyanobacteria compounds have multi-functionality and vary in their ecological roles. Lyngbyoic acid, the dominant compound isolated from IRL1, is a cyclopropane-containing fatty acid involved in enzyme inhibition that has anti-quorum-sensing activity (Kwan et al., 2011). Disruption of bacterial quorum sensing may be beneficial to the host inhibiting biofilm formation and biofouling, and controlling interactions with heterotrophic bacteria by interfering with their cell-to-cell signalling (Dobretsov et al., 2010). This strategy has also been observed in marine macroalgae (Goetze et al., 2010; Jha et al., 2013) and other marine organisms (Dobretsov et al., 2009). Malyngolide, the dominant compound isolated from IRL2, also exhibits anti-quorum sensing capabilities (Dobretsov et al., 2010). It was first isolated from *L. majuscula* by Cardellina et al. (1979) and is a known feeding deterrent to some species of fish (Thacker et al., 1997). *O. erythroflocculosa* produces microcolins (Engene et al., 2013b), which are potent immunosuppressive lipopeptides (Meickle et al., 2009). Other ecological roles of the compounds from *Lyngbya* and associated cyanobacteria include serine protease inhibition (Matthew et al., 2010), antibiotic activity (Babler et al., 1980) and growth inhibition of specific bacterial species (Cardellina et al., 1979), along with allelopathic functions to inhibit coral recruitment (Kuffner et al., 2006).

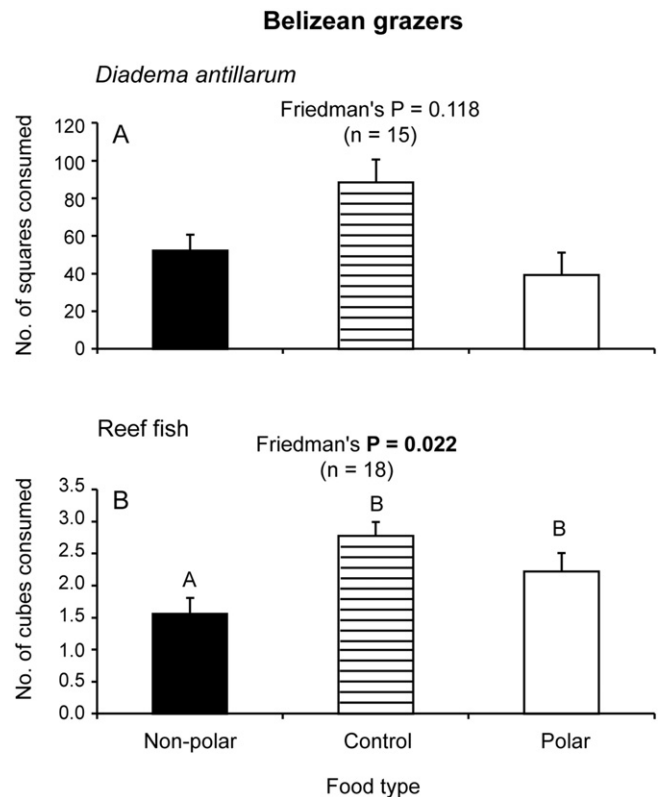
This study reiterates the specialist grazing role of *S. striatus* as a cyanobacteria consumer and also highlights their ability to show different degrees of trophic specialization likely related to secondary metabolite composition. An ability to tolerate and cope with a diverse array of multifunctional compounds in ephemeral cyanobacteria renders the sea hare at an advantage over other herbivorous organisms. Sea urchins and crabs may be well adapted to tolerate variations in cyanobacteria secondary metabolite chemistry, hence appearing indifferent, due to constraints imposed by limited mobility. However, the long term effects of prolonged cyanobacterial grazing and ability to bioaccumulate compounds have yet to be assessed in these organisms. Fish appear more sensitive to cyanobacterial secondary metabolites. However, due to their high mobility, fish are less likely to be impacted by cyanobacteria secondary metabolites than less mobile invertebrates.

Fish found in close proximity to blooms are more likely to utilize blooms to increase their rates of prey encounter and consumption due to increased numbers of mesofauna and meiofauna associated with blooms (Gilby et al., 2011) rather than utilizing the cyanobacteria itself as a food source.

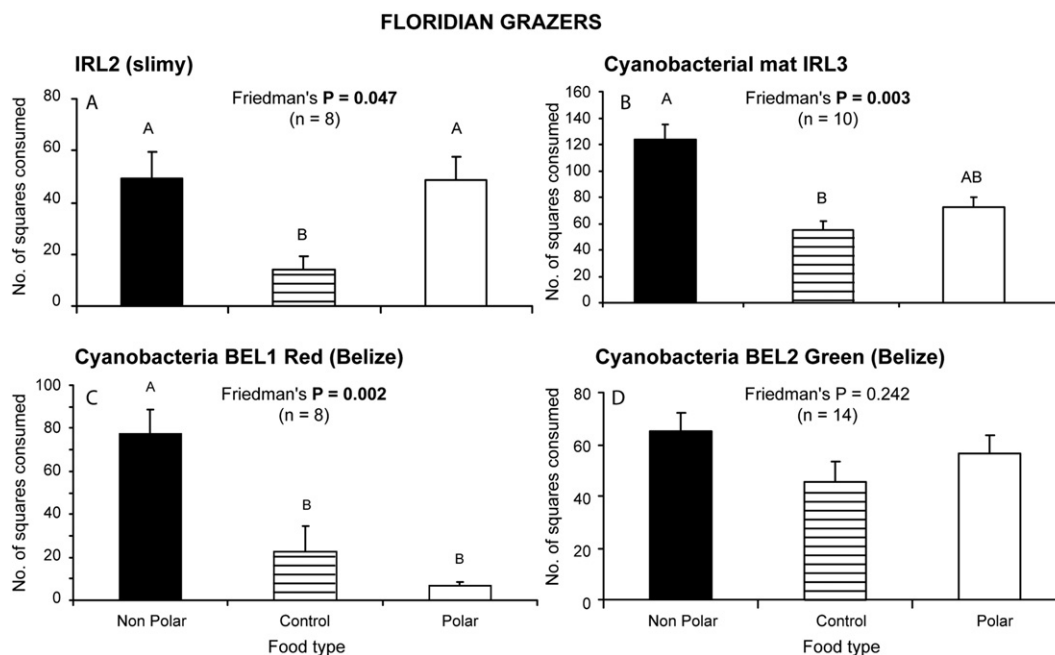
A major challenge remains for future research to untangle the chemistry associated with cyanobacterial blooms and the predictability of physiological responses in herbivorous grazers. Looking at the wider picture may obscure subtle changes happening on a smaller scale in local microhabitat environments. Further work is required to isolate compounds, leading to a more refined search for specific deterrent compounds and their impact on a local microhabitat scale vs. a larger geographic scale. This will help shape our understanding of the grazing interactions associated with cyanobacterial blooms and the ecological role of secondary metabolites.

#### 4.1. Conclusion

The focus of this study was to determine the responses of a suite of herbivores to different cyanobacterial extracts and explore the potential role of those compounds. Whilst generalist invertebrate grazers were indifferent to cyanobacterial extracts (regardless of geographical location and prior exposure), reef fish were deterred by non-polar extracts. Positive feeding cues were found in three out of four cyanobacterial extracts tested with the sea hare specialist grazer *S. striatus*. Understanding the complex interactions which exist between herbivorous grazers and cyanobacteria from tropical and sub-tropical environments and their associated chemistry is important when determining species interaction and their role within communities that have ephemeral or dominating cyanobacterial blooms.



**Fig. 4.** Palatability assay for Belizean *Okeania erythroflocculosa* crude extracts using Belizean grazers: (A) sea urchin, *Diadema antillarum* and (B) reef fish. Assays used an artificial diet of solvent treated *Gracilaria tikvahiae* (□), control; or *G. tikvahiae* with non-polar (■) or polar (□) crude extracts of *O. erythroflocculosa* treatments. Data are mean squares/cubes consumed per animal (± SE). Friedman's ANOVA was used to analyse data (A)–(E). Letters above bars denote a significant difference using a non-parametric Tukey's *post-hoc* analysis ( $P < 0.05$ ) ( $n$  = number of replicates). N.B. Scales on axes vary between graphs.



**Fig. 5.** Palatability assay for Floridian sea hare *Stylocheilus striatus* using: (A) Floridian IRL2 crude extracts; (B) Floridian cyanobacterial consortia mat IRL3 extract; (C) Belizean cyanobacteria BEL1 (red) and; (D) Belizean cyanobacteria BEL2 (green) crude extra. Assays used an artificial diet of solvent treated *Gracilaria tikvahiae* (☐), control; or *G. tikvahiae* with non-polar (■) or polar (□) crude extracts of cyanobacteria or cyanobacterial consortia sp. treatments. Data are mean squares consumed per animal ( $\pm$  SE). Friedman's ANOVA was used to analyse data. Letters above bars denote a significant difference using a non-parametric Tukey's *post-hoc* analysis ( $P < 0.05$ ). (n = number of replicates). N.B. Scales on axes vary between graphs (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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