

# Endemic grazers control benthic microalgal growth in a eutrophic tropical brackish ecosystem

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**ABSTRACT:** Anthropogenic changes to nutrient supply, numbers and behavior of grazers and interactions of these factors are known to change epilithon composition and biomass. In brackish waters, these changes occur across wide-ranging abiotic conditions (e.g. nutrient concentrations and salinity), which may alter their relative impacts on microphytobenthic communities. Such mediating processes are poorly understood, particularly in tropical brackish ecosystems. We examined the separate and interactive effects of nutrient additions and grazer exclusion on epilithon composition and biomass in eutrophic Hawaiian anchialine pools between contrasting levels of salinity and concomitant nutrient-rich groundwater influence (2.3 to 22.0 ppt; nitrite + nitrate, 6.3 to 102.6  $\mu\text{M}$ ; soluble reactive phosphorus, <0.5 to 5.18  $\mu\text{M}$ ). Across these conditions, we found no significant effects of nitrogen (N) and phosphorus (P) enrichment treatments on chlorophyll *a*, biomass (ash-free dry mass) or autotrophic index (i.e. autotrophy) of epilithon communities, and nutrient addition did not alter the effects of grazing pressure. However, autotrophy and epilithon biomass were lower in low salinity, high groundwater influenced pools, and both were strongly reduced by grazing in all pools. Furthermore, effects from grazing pressure on relative autotrophy were more pronounced in low salinity, high groundwater influenced pools. Our results suggest that (1) grazing is the primary driver of epilithon composition and biomass across these nutrient-enriched systems, (2) microphytobenthic communities in these systems are not N or P limited irrespective of co-varying salinity and background nutrients and (3) selective feeding by endemic grazers mitigates potential bottom-up forces from salinity or increased nutrient-enriched groundwater influence on autotrophy in epilithon communities.

**KEY WORDS:** Anchialine pool · Top-down control · Bottom-up control · Epilithon · Grazing · Nutrient enrichment · *Halocaridina rubra*

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## INTRODUCTION

Coastal ecosystems worldwide are experiencing changes to patterns of nutrient availability from increases in human populations, industry and urbanization and increased spread of nitrogen-fixing in-

vasive species (Vitousek et al. 1997, Cloern 2001, Compton et al. 2003). Because nitrogen (N) and phosphorus (P) commonly restrict primary production in coastal aquatic systems (i.e. 'bottom-up' control), increases in the supply of these nutrients are often responsible for significant increases in primary

production, changes in community structure of primary producers and the ultimate functioning of aquatic ecosystems (Valiela et al. 1997, Cloern 2001). Anthropogenic activities in coastal areas can also alter grazing pressure on primary producer communities (i.e. 'top-down' control) through overfishing (Burkepile & Hay 2006, Sandin et al. 2008, Williams et al. 2008, Edwards et al. 2014) or the introduction of non-native species to aquatic ecosystems (Alpine & Cloern 1992, Vander Zanden et al. 1999, Sala et al. 2011, Holitzki et al. 2013).

In nature, top-down and bottom-up forces rarely act independently (Worm et al. 2002), and the interactions of these forces on epilithon composition and biomass rather than their independent effects have become an increasing focus of investigation in various aquatic ecosystems (Rosemond et al. 1993, Hillebrand & Kahlert 2001, Smith et al. 2001, Alberti et al. 2010). For example, increases in epilithon biomass due to nutrient enrichment (bottom-up) may be followed by increased grazer density, growth and thus grazing pressure (top-down) (Rosemond et al. 1993, Nisbet et al. 1997, Hillebrand et al. 2000, Roll et al. 2005). Alternatively, grazers may show negative responses to nutrient enrichment, such as decreased growth and increased mortality rates due to shifts in epilithon composition to less desirable primary producers (Sommer 2001, Armitage & Fong 2004) or hypoxic conditions that can result from harmful algal blooms (Glibert et al. 2005, Heisler et al. 2008).

In brackish environments at the terrestrial–marine interface, anthropogenic nutrient additions and alterations to grazing pressure take place across gradients of abiotic conditions (e.g. nutrient availability, salinity and pH), which may modify their impacts on epilithon communities (Lever & Valiela 2005, Armitage et al. 2009). Understanding these interactions is of increasing importance as brackish coastal ecosystems and adjacent inland areas become increasingly populated by humans. In the tropics, coastal mixing zones are experiencing increased nutrient loading from sewage and fertilizer following resort, housing and farming development (Brock et al. 1987, Brock & Kam 1997, Wiegner et al. 2006, Bruland & MacKenzie 2010, Knee et al. 2010). These impacts will worsen as coastal human populations in the tropics continue to grow (Laws & Ferentinos 2003). Within many of these same systems, overfishing of herbivores and the introduction of exotic fish in tropical nearshore areas have also led to lowered herbivore densities (Friedlander et al. 2007, Sandin et al. 2008) and altered herbivore behavior (Capps et al. 2009, Dalton et al. 2012), respectively. Despite these

changes, studies of interactions between top-down and bottom-up forces on tropical epilithon communities remain limited and merit further investigations.

In Hawaii, anchialine pools provide unique tropical ecosystems to examine interactions of top-down and bottom-up forces on epilithon composition and communities. Anchialine pools are mixohaline environments that exhibit dampened tidal fluctuations with no surface connection to the sea (Holthuis 1973), are typically located in barren basaltic lava fields with uneven basins and are usually <0.5 m deep. The predominant faunal species in these pools is an endemic atyid shrimp, *Halocaridina rubra*, a benthic microphagous grazer thought to significantly influence anchialine pool epilithon biomass and community structure (Bailey-Brock & Brock 1993, Capps et al. 2009, Dalton et al. 2012). The highest concentration of anchialine pools in the state (>70%), along with their endemic grazers, is on the west coast of Hawai'i Island (Brock et al. 1987), an area that has experienced particularly rapid urbanization, resort development and population increases (State of Hawaii Department of Business, Economic Development and Tourism 2013). Many of the pools along this coastline have experienced increases in water nutrient concentrations and introductions of invasive fish that reduce grazing by endemic shrimp (Bailey-Brock & Brock 1993, Capps et al. 2009). However, some anchialine pools within this island subregion are considered to be relatively pristine and are protected within designated national parks and reserves.

Anchialine pools on Hawai'i Island also exist across a range of groundwater influence, and nutrient concentrations are negatively correlated with salinity across this gradient (Street et al. 2008, Knee et al. 2010, T. S. Sakihara unpubl. data). Such nutrient concentrations typically range from ca. 6 to 100  $\mu\text{M}$  of dissolved inorganic nitrogen (DIN) and ca. 0.2 to 5  $\mu\text{M}$  of soluble reactive phosphorus (SRP) across this gradient even in relatively undeveloped areas but may greatly exceed these values in areas of anthropogenic nutrient loading (ca. 120 to 180  $\mu\text{M}$  DIN and >5  $\mu\text{M}$  SRP; Wiegner et al. 2006, Dalton et al. 2012). It remains unknown whether elevated nutrient levels in undeveloped pools are a natural phenomenon or a remnant of past land use (e.g. high densities of cattle in upland areas in the 1900s; Cox 1992). Nevertheless, pools along this entire range of nutrient concentrations could not be considered pristine when related to studies of eutrophication in estuarine mixing zones worldwide (e.g. Fry et al. 2003, Lopes et al. 2007, Cloern & Jassby 2012, Barr et al. 2013) and this suggests that Hawaiian anchialine pools are nutrient enriched re-

ardless of localized anthropogenic influences. Collectively, such characteristics provide an ideal system in which to examine how interactions between grazing pressure and anthropogenic nutrient additions alter the epilithon communities of currently nutrient-enriched ecosystems (individual pools) with varying groundwater influence. Empirical investigations of nitrogen and phosphorus enrichment and density-dependent effects of grazing by *H. rubra* (i.e. relative absence of grazing) on epilithon composition across these conditions in anchialine pools are lacking.

This study examined the independent and combined effects of grazing pressure and nutrient availability on epilithon composition and biomass in Hawaiian anchialine pools across contrasting levels of groundwater influence. Our goals were to identify the impacts of (1) increased concentrations of N, P or a combination of these 2 nutrients; (2) exclusion of total grazing by *H. rubra*; and (3) the concomitant effects of these 2 factors on epilithon composition (ratio of heterotrophs to autotrophs) and biomass in Hawaiian anchialine pools between high and low levels of groundwater influence (i.e. salinity). We predicted that the contribution of autotrophs to epilithon (autotrophic index, AI) and epilithon biomass would significantly increase with nutrient enrichment treatments in high salinity pools (with lower background nutrient conditions), indicating nutrient limitation. We also predicted that epilithon biomass would significantly decrease with grazing by *H. rubra*. Lastly, we predicted that the combination of increased nutrient loading and grazing would have no effect on epilithon and biomass, presuming their independent effects would counteract each other. Our objective was to advance understanding of tropical coastal ecosystem response to altered top-down and bottom-up controls resulting from prevailing biotic and abiotic stressors.

## MATERIALS AND METHODS

### Study sites and habitat characteristics

Surveys and epilithon and water quality sampling were conducted in 11 anchialine pools across 4 sites (Kapalaoa, 'Akahukaimu, Weliweli and Manukā)

on the western coast of Hawai'i Island (Fig. 1) from September 30 to October 28, 2011. At the southernmost site at Manukā, 3 pools were sampled along the coastal boundary of the Manukā Natural Area Reserve (NAR), which was established in 1983 by the State of Hawai'i and protects 10 340 ha of undeveloped land. Because of this expansive protection and lack of development in the Manukā watershed, the pools at Manukā are considered some of the most pristine throughout the state (Brock 2004). Pools located at Kapalaoa, 'Akahukaimu and Weliweli were in a minimally developed coastal region with no development within the immediate study area. All pools were situated in relatively young (<4000 yr old) lava flows, of which the basalt substrate is close to chemically homogenous at deposition (Vitousek 1995). Sampled pools were selected based on 4 criteria that characterized natural undisturbed conditions of anchialine pools in the region and that controlled for confounding factors: (1) lack of canopy or peripheral vegetation, (2) lack of introduced or native fishes, (3) presence of an established population of *Halocaridina rubra* and (4) salinity levels consistent

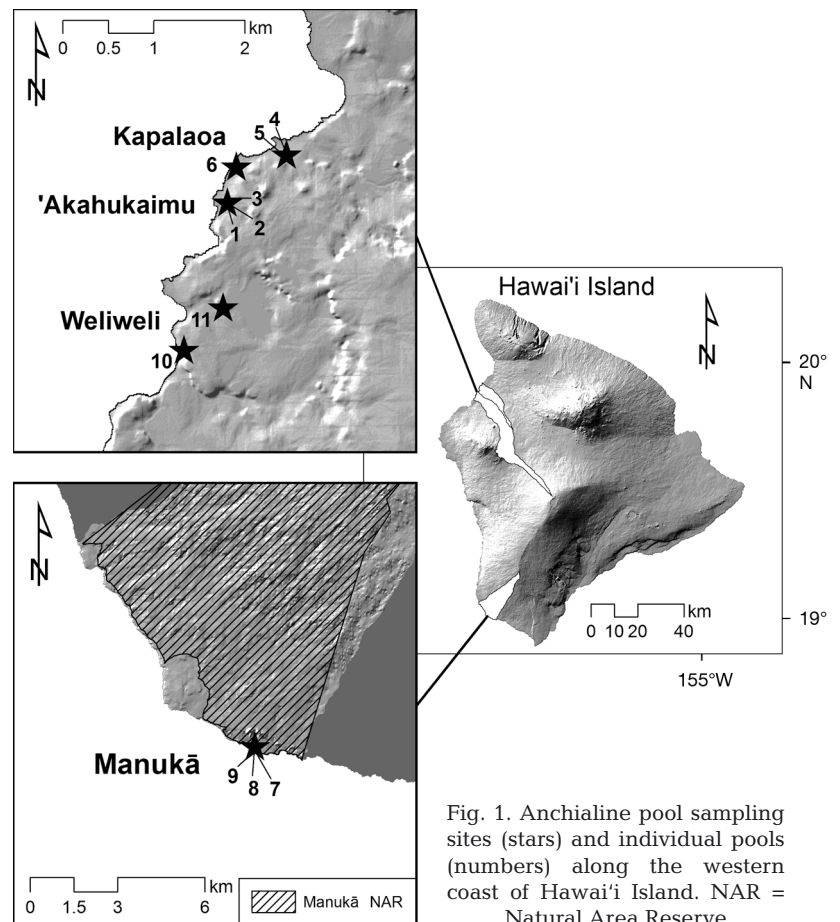


Fig. 1. Anchialine pool sampling sites (stars) and individual pools (numbers) along the western coast of Hawai'i Island. NAR = Natural Area Reserve

with background nutrient concentrations (i.e. no evidence of localized anthropogenic inputs). Concentrations of DIN and SRP in west Hawaii groundwater are substantially elevated from those in seawater, and these differences are relatively consistent throughout the western coast in the absence of anthropogenic loading, resulting in linear relationships between salinity and nutrient concentrations within mixing zones (Street et al. 2008, Knee et al. 2010). As a result, a great majority of the variation in the background nutrient environment between pools in this study can be attributed to the proportion of pool water that is derived from groundwater.

Measurements of physiochemical parameters and habitat characteristics of each sampled pool were conducted immediately prior to placement of experimental treatments. Measurements included water temperature ( $^{\circ}\text{C}$ ), salinity (ppt), conductivity ( $\text{mS cm}^{-1}$ ), pH, dissolved oxygen ( $\text{mg l}^{-1}$ ), turbidity (NTU), water depth (cm), pool surface area ( $\text{m}^2$ ) and pool type (single pool or pool complex). As these pools are physically dynamic through tidal cycles, our measurements were characteristic of conditions at low tide with respect to water depth, water temperature, pH and dissolved oxygen, which are typically higher at high tides (Dudley et al. in press). However, salinity gradients in these pools are relatively constant spatially and temporally (Brock et al. 1987, Dudley et al. in press), while vertical salinity profiles in shallower pools are frequently uniform (Havird et al. 2014).

### Experimental design and methodology

Microalgae were sampled using 8 cm diameter unglazed terra-cotta plates, which were used as algae recruitment tiles (Pringle & Triska 2006). Nine plates (an experimental array) were deployed in each pool for 29 d to concurrently examine the effects of 2 predictor variables (nutrient loading and grazing pressure) and their interactions on epilithon biomass and nutrient stoichiometry. A 'grazer exclusion' and an 'open' plate were each assigned one of 4 nutrient enrichment treatments (N, P, N+P, and a control with no nutrient enrichment), for a total of 8 plates. Nutrient enrichment was accomplished by using diffusion media composed of a 3 % agar solution that was either 5 M nitrate ( $\text{NaNO}_3$ ) for N treatments, 1 M phosphate

( $\text{KH}_2\text{PO}_4$ ) for P treatments or 5 M  $\text{NaNO}_3$  + 1 M  $\text{KH}_2\text{PO}_4$  for N+P treatments (Tank et al. 2006). Control treatments were comprised of only 3 % agar solution. A volume of 60 ml of each mixture was poured into separate plates and left for a few hours to set. To ensure a unidirectional diffusion of nutrients through the undersurface of the plate, a 10 cm diameter plastic petri dish cover was placed over the agar and sealed around the edges with silicone. Grazer exclusion plates were designed to cover the diffusing surface of each plate with a grazer exclusion cage constructed of 500  $\mu\text{m}$  Nitex® bolting cloth glued to a 10 cm diameter plastic petri dish frame (Fig. 2). Plates open to grazers were similarly constructed, except with 4 openings ( $3.2 \text{ cm}^2$ ) on the sides of the frame to allow grazers onto the diffusing surface (Fig. 2). The remaining ninth terra-cotta plate did not have any diffusion media or exclusion cage and functioned as a procedural control to address potential confounding effects of the grazer exclusion hardware or agar media on benthic algal production or grazing pressure.

All plates were placed with the diffusing surface facing up in areas of the pools that were exposed to sunlight and were constantly submerged under water. No anchoring or securing system was used to minimize any disturbance to natural conditions and because water conditions are perpetually calm in these pools. Plates with the different nutrient treatments were placed at a minimum distance of 1 m apart to reduce confounding effects across nutrient treatments. To verify that nutrients were diffusing throughout the duration of the experiment, 15 ml water samples were collected for nutrient concentration analyses directly above the surface of the plates 2 h after deployment and immediately before the plates were removed (29 d).

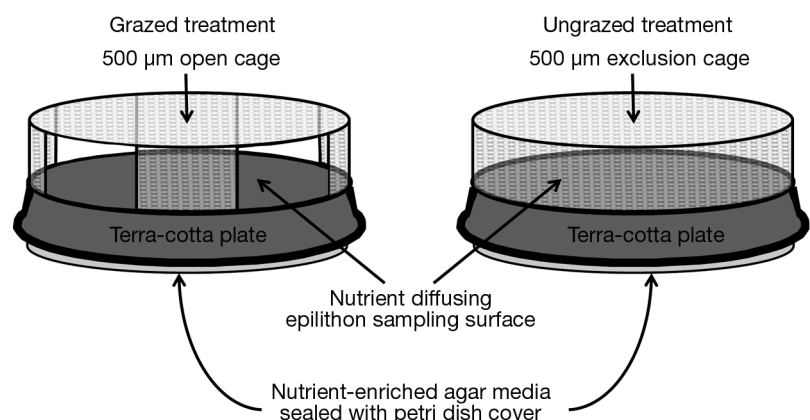


Fig. 2. Schematic drawing of unglazed terra-cotta epilithon samplers



Additionally, water samples were collected from each pool approximately 2 to 3 m away from the plates to measure background nitrite + nitrate ( $\text{NO}_2^- + \text{NO}_3^-$ ) (hereon referred to as  $\Sigma\text{NO}_3^-$ ), SRP and ammonium ( $\text{NH}_4^+$ ) concentrations.  $\Sigma\text{NO}_3^-$  and SRP were the nutrients of interest included in the enrichment treatments, as their concentrations have more than doubled in anchialine pools within areas of increased development over the past few decades (Wiegner et al. 2006). All water samples were filtered through 0.7  $\mu\text{m}$  (GF/F) filters and analyzed for  $\Sigma\text{NO}_3^-$  (detection limit [DL]: 0.1  $\mu\text{M}$ , USEPA 353.4), SRP (DL: 0.1  $\mu\text{M}$ , USEPA 365.5) and  $\text{NH}_4^+$  (DL: 1  $\mu\text{M}$ , USGS I-2525) using a Pulse Technicon II<sup>TM</sup> autoanalyzer.

### Visual surveys of grazers

Densities of *H. rubra* and other benthic grazers, as well as community composition of benthic invertebrates, were estimated by visual counts using quadrats (0.06 m<sup>2</sup>), similar to methods by Havird et al. (2013). Each pool was systematically surveyed along a transect line, which was located along the longest length across the pool. Four visual counts within the quadrat were conducted equidistantly along the transect line. Each quadrat was placed on the bottom and left undisturbed for 1 min, after which visual counts of all benthic motile animals located within the quadrat area were recorded. This proved to be the most efficient and unbiased method of estimating grazer density, as the pools' sharp, uneven basaltic substrata were not conducive for sampling with hand nets or potentially biased baited-trap methods. These data verified the presence of the dominant grazer, *H. rubra*, and calculated average densities were used as a measure of grazing pressure on periphyton in each pool. Significant diel vertical migrations between cryptic and exposed anchialine habitat have been documented in *H. rubra* (Capps et al. 2009, Carey et al. 2011, Dalton et al. 2012, Sakihara 2012). Therefore, to ensure consistent estimates of *H. rubra* density, visual counts were conducted during the day and night within the same 24 h tidal cycle and tidal period (e.g.  $\pm 2$  h of low tide).

### Sample processing and laboratory analysis

After 29 d of deployment, all algal samplers were removed from the pools and processed in the field.

The flat 53.5 cm<sup>2</sup> diffusing surface of each sampler was gently scrubbed clean with a stiff-bristled brush for 1 min and rinsed into a slurry of known volume with water from the sampled pool that had been filtered through a 0.7  $\mu\text{m}$  GF/F filter. Slurry samples were stored in the dark and on ice (4°C; Pringle & Triska 2006) and returned to the laboratory, where they were immediately processed. Subsamples of the aliquot were vacuum filtered through pre-combusted 0.7  $\mu\text{m}$  GF/F filters. Separate subsamples were processed for analyses of chlorophyll *a* (chl *a*); ash-free dry mass (AFDM); and particulate carbon (C), N and P (Hillebrand & Kahlert 2001).

Epilithon chl *a* content, a measure of the autotrophic component of epilithon biomass, was quantified using a Turner Designs<sup>TM</sup> (model 10AU) fluorometer (Arar & Collins 1997). Chl *a* was extracted from the filtered samples in 5 ml of 90% buffered acetone at -15°C for 24 h. Initial fluorometric readings were recorded, and then each sample was acidified by adding 3 drops of 0.1 N HCl and allowed to steep for 1 min. A second fluorometric reading was then recorded and used to calculate chl *a* concentrations by correcting for phaeophytin. AFDM samples, a measure of epilithon biomass (i.e. both the autotrophic and heterotrophic component), were filtered on pre-weighed 0.7  $\mu\text{m}$  GF/F filters and then dried for 48 h at 60°C. The filters were then weighed on an analytical microbalance (to the nearest 1.0  $\mu\text{g}$ ), ashed at 500°C for 4 h and reweighed. AFDM concentration was then calculated by methods described by Steinman et al. (2006). An AI (AFDM chl *a*<sup>-1</sup>) was calculated to assess the trophic condition of the epilithon community by indicating relative shifts towards autotrophy or heterotrophy. A higher AI indicated relative heterotrophy, whereas a lower AI indicated relative autotrophy (Steinman et al. 2006).

Particulate C and N filtered samples were dried for 48 h at 60°C, rolled in tin boats and combusted in a Costech elemental analyzer. C:N molar ratios were calculated based on respective molar mass measurements of C and N. Particulate P was measured using AFDM filter samples, which were processed based on methods described by Hill & Fanta (2008). The P samples were analyzed on a Varian Vista MPX ICP-OES spectrometer. Sufficient biomass for particulate P analysis was only present in ungrazed treatment samples, and therefore no particulate P results are available for grazed treatments. N:P and C:P molar ratios were then calculated for ungrazed treatment samples based on molar mass measurements of C, N and P.

### Data analyses

One-way analysis of variance (ANOVA) tests were used to compare  $\Sigma\text{NO}_3^-$  and SRP concentrations of water samples taken from the diffusing plate surfaces among nutrient treatments and background concentrations followed by a post hoc Tukey's HSD test to verify effective N and P diffusion for desired treatments. In addition, 1-way ANOVA tests were used to compare AFDM, chl *a*, AI and C:N ratios between procedural control treatments and control treatments to address potential confounding effects of grazer exclusion hardware or agar media. One-way ANOVA tests were also used to compare day and night density estimates of *H. rubra* within pools. All response variables were natural log transformed to meet the assumptions of normality and homogeneity of variances. Examination of the transformed data showed no significant violations to the assumptions of a 1-way ANOVA test. Pearson product-moment correlation was used to examine the relationship between background  $\Sigma\text{NO}_3^-$  and SRP concentrations and between *H. rubra* densities and epilithon measurements (i.e. chl *a*, AFDM, AI and C:N ratios). Values were log transformed to meet the assumptions of a Pearson product-moment correlation. Spearman's rank correlations were used to examine relationships between physiochemical parameters (i.e. water temperature, salinity, conductivity, dissolved oxygen, turbidity), background  $\Sigma\text{NO}_3^-$  concentrations, SRP and *H. rubra* densities when assumptions for a Pearson product-moment correlation were not met. Results  $p \leq 0.05$  were reported as significant.

Three-factor ANOVA tests with interactions were used to examine the effects of grazing treatments,

nutrient additions and salinity level on AFDM, chl *a*, AI and C:N ratios. Significant correlations were found between salinity and background  $\Sigma\text{NO}_3^-$  concentrations ( $r = -0.88$ ,  $p < 0.001$ ) in anchialine pool surveys conducted in October and November 2010, similar to those seen in anchialine pools by Street et al. (2008) and Knee et al. (2010). Conductivity and pH would also be expected to vary predictably across this salinity gradient (Wong 1979). In addition, background SRP and  $\text{NH}_4^+$  concentrations are known to be positively correlated with  $\Sigma\text{NO}_3^-$  concentrations in these ecosystems (Dalton et al. 2012). Therefore, to simplify the ANOVA model, salinity level served as a surrogate measure for parameters associated with groundwater influence ( $\Sigma\text{NO}_3^-$ , SRP,  $\text{NH}_4^+$ , conductivity and pH). N and P additions, grazing pressure, salinity and their interactions as drivers of our response variables were examined. Nutrient addition was examined at 4 levels (N, P, N+P, control), grazing was examined at 2 levels (grazed, ungrazed) and salinity was examined at 2 levels (high:  $\geq 17.5$  ppt,  $n = 3$ ; and low:  $< 17.5$  ppt,  $n = 8$ ) based on the well-separated bimodal distribution of salinity measured in our pools (Table 1). Because this division resulted in unbalanced replication in our 2 salinity factor levels, we used type II sums of squares for these ANOVA tests. All response variables were Box-Cox transformed to meet or improve the assumptions of ANOVA.

Two-factor ANOVA tests with interactions were used to explicitly examine the separate effects and interactions of nutrient treatment and salinity level on C:N, C:P and N:P molar ratios of ungrazed treatments. Dependent variables that did not meet the assumptions of ANOVA tests were Box-Cox transformed (C:P and N:P).  $p$ -values for all 2- and 3-factor ANOVA

Table 1. Physical and physiochemical characteristics and *Halocaridina rubra* density estimates of each pool. Measurements not recorded indicated by '–'

Site	Pool	Surface area (m <sup>2</sup> )	Maximum depth (m)	Temperature (°C)	Salinity (ppt)	Conductivity (mS cm <sup>-1</sup> )	Dissolved oxygen (mg l <sup>-1</sup> )	pH	Mean <i>H. rubra</i> ind. m <sup>-2</sup> ± SE
'Akahukaimu	1	25.1	0.69	25.1	3.9	7.1	8.00	–	158 ± 38.7
	2	66.2	0.62	25.2	3.1	5.8	8.76	–	392 ± 60.5
	3	228.0	1.39	25.7	3.8	7.0	8.13	–	277 ± 42.1
Kapalaoa	4	1.0	–	24.5	4.0	7.3	7.75	–	1293 ± 430.5
	5	1.4	0.15	25.9	4.2	7.6	7.69	–	1797 ± 444.8
	6	4.2	0.66	24.4	3.9	7.0	8.16	7.96	287 ± 135.0
Manukā	7	17.5	0.57	24.5	22.0	–	5.88	9.20	1554 ± 483.1
	8	124.6	0.64	24.6	21.0	–	6.33	9.21	289 ± 125.5
	9	46.8	0.55	24.6	20.0	–	6.26	8.74	1235 ± 522.2
Weliweli	10	6.4	0.51	23.4	2.8	5.2	5.20	7.96	194 ± 21.3
	11	31.0	0.94	23.9	2.3	4.3	7.99	7.56	258 ± 38.3

analyses were adjusted to control for multiple comparisons using the false discovery rate (FDR) method (Benjamini & Hochberg 1995). Significant results were reported on FDR-adjusted  $p$ -values  $\leq 0.05$ . All statistical tests were conducted using R statistical software version 3.0.1 (R Development Core Team 2010). All error terms are reported as  $\pm 1$  standard error.

## RESULTS

### Habitat characteristics and grazer density

Pool characteristics varied considerably among sampling sites. Pool surface area ranged from a 1 m<sup>2</sup> pool complex to a 228 m<sup>2</sup> single pool (Table 1). Background  $\Sigma\text{NO}_3^-$  concentrations ranged from 6.37 to 102.64  $\mu\text{M}$  between pools (Table 2) and were negatively correlated with salinity ( $\rho = -0.86$ ,  $p < 0.001$ ), ranging from 2.3 to 22.0 ppt (Table 1), and pH ( $\rho = -0.99$ ,  $p < 0.001$ ), ranging from 7.56 to 9.21 (Table 1). A positive correlation was also found between background  $\Sigma\text{NO}_3^-$  and SRP concentrations ( $r = 0.84$ ,  $p = 0.001$ ). No other correlations were found between physical parameters, background nutrient concentrations or *Halocaridina rubra* densities.

Average *H. rubra* densities ( $\pm$  SE) ranged from  $158.0 \pm 38.7$  to  $1797.0 \pm 444.8$  ind. m<sup>-2</sup> across all pools (Table 1). Less common benthic invertebrates were also observed, such as *Metabetaeus lohena* ( $3.0 \pm 0.8$  ind. m<sup>-2</sup>) and amphipods ( $0.4 \pm 0.4$  ind. m<sup>-2</sup>). Nonetheless, *H. rubra* was the dominant species present in all pools, and no differences in average densities were detected between day and night surveys (day:  $759.1 \pm 138.9$  ind. m<sup>-2</sup>; night:  $505.7 \pm 138.8$  ind. m<sup>-2</sup>). Variances of all response variables and the effect of grazing by *H. rubra* were best explained by using a categorical grazed/ungrazed predictor in the statistical models compared to using continuous grazer density estimates.

### Nutrient diffusion and grazer exclusion

Concentrations of  $\Sigma\text{NO}_3^-$  ( $\mu\text{M}$ ) among the 4 nutrient treatments and background concentrations were significantly different at the start of the experiment (Day 1, all stations pooled; ANOVA,  $F_{4,94} = 20.5$ ,  $p < 0.001$ ). Concentrations of  $\Sigma\text{NO}_3^-$  were significantly higher in N and N+P treatments than in all other treatments and background concentrations (Tukey's HSD,  $p < 0.001$ ), whereas  $\Sigma\text{NO}_3^-$  in P, controls and background samples were not significantly different

from each other. Analysis of water samples at the end of the experiment (Day 29) was consistent with the initial results ( $F_{4,94} = 8.4$ ,  $p < 0.001$ ), with significantly higher  $\Sigma\text{NO}_3^-$  in N and N+P treatments compared to other nutrient treatments and background concentrations (Tukey's HSD,  $p < 0.02$ ). As observed for N treatments, SRP concentrations were significantly higher in P and N+P treatments (Tukey's HSD, Day 1:  $p \leq 0.002$ , Day 29:  $p < 0.02$ ), whereas SRP in N, controls and background samples were not significantly different from each other.

No differences were found between procedural control and control treatments for all response variables (Table A1 in the Appendix). The open grazer exclusion hardware and agar media were thereby confirmed to have no confounding effects on the response variables. Procedural controls were not included in the statistical tests presented in the autotrophy and C:N:P stoichiometry results sections to improve the balance of our ANOVA designs.

### Nutrient and grazing effects on epilithon

A main effect on chl *a* concentrations from grazing treatment was observed (3-factor ANOVA,  $F_{1,72} = 179.03$ , FDR-corrected  $p < 0.001$ , Fig. 3A). Average chl *a* concentrations were 10 times greater on ungrazed treatments than on grazed treatments. A main effect on chl *a* concentrations from salinity level was also observed (3-factor ANOVA,  $F_{1,72} = 68.4$ , FDR-corrected  $p < 0.001$ ). Greater chl *a* concentrations were observed in high salinity pools for both grazing treatments (Fig. 3A). No main effects from nutrient treatments were found, although in low salinity-ungrazed treatments, chl *a* concentrations were at least 2 times higher in N and N+P treatments compared to control and P treatments (Fig. 3A). In high salinity-ungrazed treatments, chl *a* was 3 to 5 times lower in N and P treatments than control and N+P treatments (Fig. 3A). No interactive effects on chl *a* concentrations were found between grazing treatment, salinity and nutrient treatment based on FDR-corrected  $p$ -values (Table A2 in the Appendix). No correlations were found between *H. rubra* densities and chl *a* concentrations ( $r = 0.01$ ,  $p = 0.97$ ).

Similarly, a main effect on AFDM from grazing treatment was observed (3-factor ANOVA,  $F_{1,72} = 139.6$ , FDR-corrected  $p < 0.001$ , Fig. 3B). Average AFDM values were nearly 4 times greater on ungrazed treatments than on grazed treatments. A main effect from salinity was also observed (3-factor ANOVA,  $F_{1,72} = 79.2$ , FDR-corrected  $p < 0.001$ ).

Table 2. Nutrient concentrations ( $\mu\text{M}$ ) nitrite + nitrate ( $\Sigma\text{NO}_3^-$ ), soluble reactive phosphorus (SRP) and background ammonium ( $\text{NH}_4^+$ ) at the start and end of the experiment, September 30 to October 28, 2011. Background  $\Sigma\text{NO}_3^-$ , SRP and  $\text{NH}_4^+$  are presented. Mean ( $\pm$  SE)  $\Sigma\text{NO}_3^-$  and SRP are presented for controls and nutrient enrichment treatments nitrogen (N), phosphorus (P) and nitrogen + phosphorus (N+P). ND = no detection. Measurements not recorded indicated by '-'. Measurements in parentheses indicate single absolute values

Site	Pool	Background		Control			N			Treatment			P			N+P		
		$\Sigma\text{NO}_3^-$	SRP	$\text{NH}_4^+$	$\Sigma\text{NO}_3^-$	SRP	$\Sigma\text{NO}_3^-$	SRP	$\Sigma\text{NO}_3^-$	SRP	$\Sigma\text{NO}_3^-$	SRP	$\Sigma\text{NO}_3^-$	SRP	$\Sigma\text{NO}_3^-$	SRP		
DAY 1																		
'Akahukaimu	1	(73.6)	(2.2)	(1.4)	75.5 $\pm$ 1.2	2.0 $\pm$ 0.1	252.9 $\pm$ 170.8	1.9 $\pm$ 0.02	72.3 $\pm$ 1.8	30.2 $\pm$ 9.1	239.7 $\pm$ 88.3	8.2 $\pm$ 1.6						
	2	(79.6)	(1.5)	(1.0)	79.2 $\pm$ 0.3	1.4 $\pm$ 0.1	99.1 $\pm$ 15.6	1.5 $\pm$ 0.04	78 $\pm$ 1.2	6.9 $\pm$ 1.1	177.4 $\pm$ 56.9	15.9 $\pm$ 5.8						
	3	(73.8)	(1.9)	(1.3)	72.8 $\pm$ 0.9	1.9 $\pm$ 0.05	283.7 $\pm$ 161.3	1.5 $\pm$ 0.05	71.7 $\pm$ 0.4	26.1 $\pm$ 11.9	178.8 $\pm$ 59.5	19.8 $\pm$ 7.6						
Kapalaoa	4	(61.2)	(1.9)	–	63.8 $\pm$ 4.5	2.1 $\pm$ 0.1	390.7 $\pm$ 141.5	4.0 $\pm$ 0.5	60.5 $\pm$ 0.4	27.7 $\pm$ 4.9	151.7 $\pm$ 49.7	12.7 $\pm$ 4.6						
	5	(102.6)	(5.2)	(1.6)	93.2 $\pm$ 5.9	3.1 $\pm$ 1.3	157.3 $\pm$ 16.9	1.9 $\pm$ 0.03	72.3 $\pm$ 4.4	16.8 $\pm$ 2.4	224.0 $\pm$ 71.9	30.7 $\pm$ 10.6						
	6	(65.9)	(1.8)	(1.7)	65.1 $\pm$ 0.5	1.9 $\pm$ 0.08	334.2 $\pm$ 88.0	2.2 $\pm$ 0.1	64.8 $\pm$ 0.2	58.4 $\pm$ 24.4	153.3 $\pm$ 49.5	10.7 $\pm$ 3.8						
Manukā	7	(11.4)	(0.2)	ND	8.7 $\pm$ 1.6	0.2 $\pm$ 0.05	154.1 $\pm$ 13.8	ND	38.7 $\pm$ 17.8	45.4 $\pm$ 1.4	300.7 $\pm$ 108.6	11.4 $\pm$ 3.5						
	8	(6.4)	(0.0)	ND	7.4 $\pm$ 0.6	(0.4)	94.8 $\pm$ 17.6	(0.7)	9.5 $\pm$ 2.3	51.9 $\pm$ 20.9	177.3 $\pm$ 41.3	6.4 $\pm$ 0.5						
	9	(8.8)	(1.2)	ND	8.3 $\pm$ 0.5	0.7 $\pm$ 0.3	201.6 $\pm$ 112.0	(0.1)	8.7 $\pm$ 0.9	120.8 $\pm$ 27.0	794.3 $\pm$ 724.3	11.9 $\pm$ 4.7						
Weliweli	10	(73.9)	(1.1)	(0.1)	73.3 $\pm$ 1.1	1.1 $\pm$ 0.1	528.7 $\pm$ 343.9	1.2 $\pm$ 0.03	72.9 $\pm$ 0.5	16.0 $\pm$ 2.2	153.6 $\pm$ 43.9	6.9 $\pm$ 2.7						
	11	(87.2)	(1.4)	(0.8)	87.5 $\pm$ 0.2	1.4 $\pm$ 0.03	376.9 $\pm$ 49.7	1.3 $\pm$ 0.08	84.3 $\pm$ 0.2	39.5 $\pm$ 12.7	299.8 $\pm$ 31.6	23.3 $\pm$ 9.3						
DAY 29																		
'Akahukaimu	1	(74.5)	(1.8)	–	74.1 $\pm$ 0.2	1.8 $\pm$ 0.01	78.4 $\pm$ 0.2	1.8 $\pm$ 0.005	73.4 $\pm$ 0.02	4.5 $\pm$ 1.2	108.6 $\pm$ 7.6	7.2 $\pm$ 1.7						
	2	(67.6)	(1.6)	–	72.4 $\pm$ 3.0	1.7 $\pm$ 0.07	116.4 $\pm$ 18.8	1.7 $\pm$ 0.07	68.7 $\pm$ 0.07	5.1 $\pm$ 0.3	81.3 $\pm$ 0.4	2.3 $\pm$ 0.1						
	3	(72.1)	(2.0)	–	71.1 $\pm$ 0.8	1.9 $\pm$ 0.06	98.2 $\pm$ 11.3	1.8 $\pm$ 0.001	70.1 $\pm$ 0.2	4.2 $\pm$ 0.1	87.3 $\pm$ 0.2	3.5 $\pm$ 0.5						
Kapalaoa	4	(55.4)	(2.1)	–	52.8 $\pm$ 3.6	2.1 $\pm$ 0.1	61.9 $\pm$ 3.7	2.2 $\pm$ 0.09	33.7 $\pm$ 1.5	9.1 $\pm$ 0.6	67.3 $\pm$ 2.4	4.9 $\pm$ 0.4						
	5	(39.7)	(1.7)	–	34.3 $\pm$ 5.7	1.6 $\pm$ 0.3	273.8 $\pm$ 98.3	1.9 $\pm$ 0.004	42.2 $\pm$ 11.4	4.2 $\pm$ 0.3	471.4 $\pm$ 138.9	79.9 $\pm$ 33.5						
	6	(60.3)	(2.1)	–	60.7 $\pm$ 0.6	2.1 $\pm$ 0.03	418.0 $\pm$ 173.7	3.1 $\pm$ 0.1	43.5 $\pm$ 0.3	6.7 $\pm$ 0.2	606.3 $\pm$ 257.1	180.7 $\pm$ 88.5						
Manukā	7	(8.4)	(0.5)	–	8.2 $\pm$ 0.7	0.4 $\pm$ 0.1	80.7 $\pm$ 31.7	0.2 $\pm$ 0.1	7.5 $\pm$ 1.3	2.9 $\pm$ 0.6	74.6 $\pm$ 16.5	2.4 $\pm$ 0.5						
	8	(10.5)	(0.6)	–	10.5 $\pm$ 0.2	0.6 $\pm$ 0.02	291.4 $\pm$ 125.3	0.6 $\pm$ 0.2	11.1 $\pm$ 0.5	1.9 $\pm$ 0.6	254.3 $\pm$ 107.0	6.2 $\pm$ 2.4						
	9	(11.6)	(1.0)	–	10.3 $\pm$ 1.3	0.9 $\pm$ 0.2	57.3 $\pm$ 19.4	0.9 $\pm$ 0.06	11.3 $\pm$ 0.3	4.7 $\pm$ 1.5	368.1 $\pm$ 143.5	6.6 $\pm$ 1.8						
Weliweli	10	(74.0)	(1.8)	–	73.4 $\pm$ 0.5	1.5 $\pm$ 0.2	101.6 $\pm$ 13.2	1.3 $\pm$ 0.004	71.9 $\pm$ 0.2	4.4 $\pm$ 1.4	107.3 $\pm$ 18.0	2.8 $\pm$ 0.7						
	11	(88.9)	(1.4)	–	88.3 $\pm$ 0.4	1.5 $\pm$ 0.02	108.4 $\pm$ 4.9	1.5 $\pm$ 0.09	82.5 $\pm$ 0.2	5.9 $\pm$ 1.0	110.9 $\pm$ 9.9	6.0 $\pm$ 2.0						



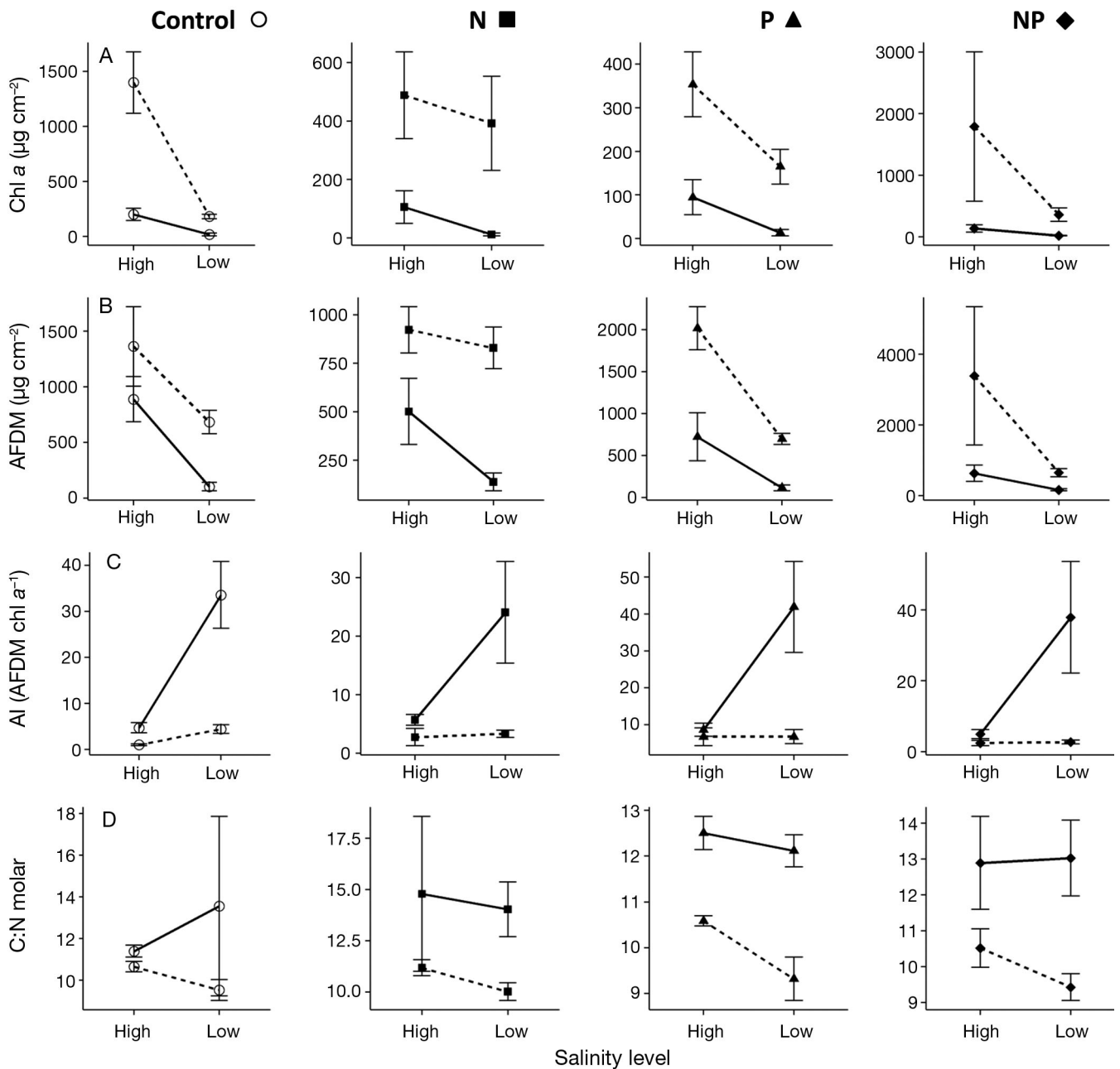


Fig. 3. Interaction plots of microalgal response measurements for grazed (solid line) and ungrazed (dashed line) treatments vs. salinity level (high:  $\geq 20$  ppt, low:  $< 5$  ppt). Columns are sorted by nutrient treatments: control, nitrogen (N), phosphorus (P) and nitrogen + phosphorus (NP). (A) Chlorophyll *a* (chl *a*,  $\mu\text{g cm}^{-2}$ ), (B) ash-free dry mass (AFDM,  $\mu\text{g cm}^{-2}$ ), (C) autotrophic index (AI, AFDM chl *a*<sup>-1</sup>), (D) carbon:nitrogen (C:N) ratio. Shapes are mean values, and error bars are  $\pm 1$  standard error

Greater AFDM values were observed in high salinity pools for both grazing treatments (Fig. 3B). No interactive effects were found between grazing treatments, salinity and nutrient treatments on AFDM (Table A2 in the Appendix). No correlations were found between *H. rubra* densities and AFDM ( $r = -0.01$ ,  $p = 0.97$ ).

Grazing treatment also had a main effect on AI (3-factor ANOVA,  $F_{1,72} = 89.9$ , FDR-corrected  $p < 0.001$ , Fig. 3C). AI was nearly 7 times lower for ungrazed treatments compared to grazed treatments. In addition, a main effect from salinity was found (3-factor ANOVA,  $F_{1,72} = 19.7$ , FDR-corrected  $p < 0.001$ ), as AI averages were lower in high salinity pools for

both grazing treatments. This indicates that the proportion of epilithon comprised of autotrophic biomass was significantly decreased by grazing and was consistently less in low salinity pools. No significant interaction was found between grazing treatments and salinity, although differences in AI between grazing treatments seemed to be more pronounced in low salinity pools (Fig. 3C). No other interaction effects and no main effects of nutrient treatments on AI were found (Table A2 in the Appendix). No correlation was found between *H. rubra* densities and AI ( $r = 0.04$ ,  $p = 0.91$ ).

Grazing treatments had a main effect on C:N molar ratios (3-factor ANOVA,  $F_{1,72} = 18.4$ , FDR-corrected  $p < 0.001$ , Fig. 3D). On grazed treatments, C:N averaged  $13.1 \pm 0.8$  vs.  $9.9 \pm 0.2$  on ungrazed treatments. No other main effects or interactions between grazing treatment, salinity and nutrient treatments on C:N were found (Table A2 in the Appendix). No correlation was found between *H. rubra* densities and C:N ( $r = -0.23$ ,  $p = 0.50$ ).

### C:N:P stoichiometry

Salinity had a main effect on C:N (2-factor ANOVA,  $F_{1,36} = 8.8$ , FDR-corrected  $p < 0.02$ , Fig. 4A) and N:P (2-factor ANOVA,  $F_{1,36} = 4.4$ , FDR-corrected  $p < 0.05$ , Fig. 4C) molar ratios across ungrazed treatments. No main effects or interactive effects were found on C:P (Fig. 4B). No main effects from nutrient treatments or interactive effects were found for C:N and N:P (Table A3 in the Appendix).

## DISCUSSION

Anthropogenic influences on combined top-down and bottom-up controls of primary productivity in coastal ecosystems is a standing global issue that has long provided impetus for investigating trophic interactions and ecosystem dynamics. For the first time in a tropical estuarine system, we investigated the interactions of N and P additions and grazing on epilithon communities in systems that differ in levels of groundwater influence.

Over the duration of the experiment, the nutrient diffusion media enriched the sampling surface of the terra-cotta plates with the desired nutrients (N, P and N+P) by more than 2 times the background water concentrations. In addition, overdifusion of nutrients across treatments and throughout the pool was not observed. Grazer exclusion cages successfully pre-

vented *Halocaridina rubra* from grazing the sampled surfaces, while active grazing was observed on open plates. Potential shading effects and impeded water flow from the exclusion cages were not concerns, as the cages were virtually free of fouling after 29 d. This was verified by the lack of differences in all response variables between procedural control and control samples. The lack of effects from our nutrient diffusion plates on chl *a* or AFDM did not support our first hypothesis of significant nutrient limitation for these anchialine pools and revealed that differences in epilithon composition between salinity levels—as evidenced by change in AI—were not driven by co-varying nutrient concentrations. The significant reduction in chl *a* and AFDM by grazing, however, fully supported our second hypothesis of significant top-down forces from grazing. Finally, the lack of significant interactions between grazing treatments, salinity and nutrient treatments did not support our third hypotheses that we would observe combined top-down and bottom-up control on epilithon. Instead, other factors that are influenced by groundwater besides background  $\Sigma\text{NO}_3^-$  and SRP concentrations, such as salinity, may be drivers of the autotrophic composition and biomass of epilithon communities. Chl *a* concentrations and epilithon AFDM were lower in pools with low salinity (more groundwater influence), and epilithon was more heterotrophic (higher AI) on grazed treatments and in lower salinity, suggesting that grazers exert a strong top-down control on autotrophy, and their effects on epilithon community structure was most discernible where autotrophic species were least abundant.

### Abiotic limitations on epilithon

The lack of nutrient treatment effects on epilithon biomass and autotrophy was in contrast to our first hypothesis. We predicted that nutrient limitation would be a primary constraint of standing autotrophic biomass; thus, epilithon communities in high salinity (relative lower  $\Sigma\text{NO}_3^-$  and SRP concentration) would be more responsive to nutrient additions. This may be a result of relatively high background  $\Sigma\text{NO}_3^-$  and SRP concentrations in these high salinity pools (6.4 to 11.6  $\mu\text{M}$   $\Sigma\text{NO}_3^-$  and <0.5 to 1.2  $\mu\text{M}$  SRP). While these values were at the lower end of the background nutrient spectrum measured across all 11 pools, they are still well above the geometric means recommended for estuarine waters by the Hawaii Department of Health (0.5  $\mu\text{M}$   $\Sigma\text{NO}_3^-$  and 0.8  $\mu\text{M}$  total P; HDOH 2004). Nutrient availability in these pools may there-

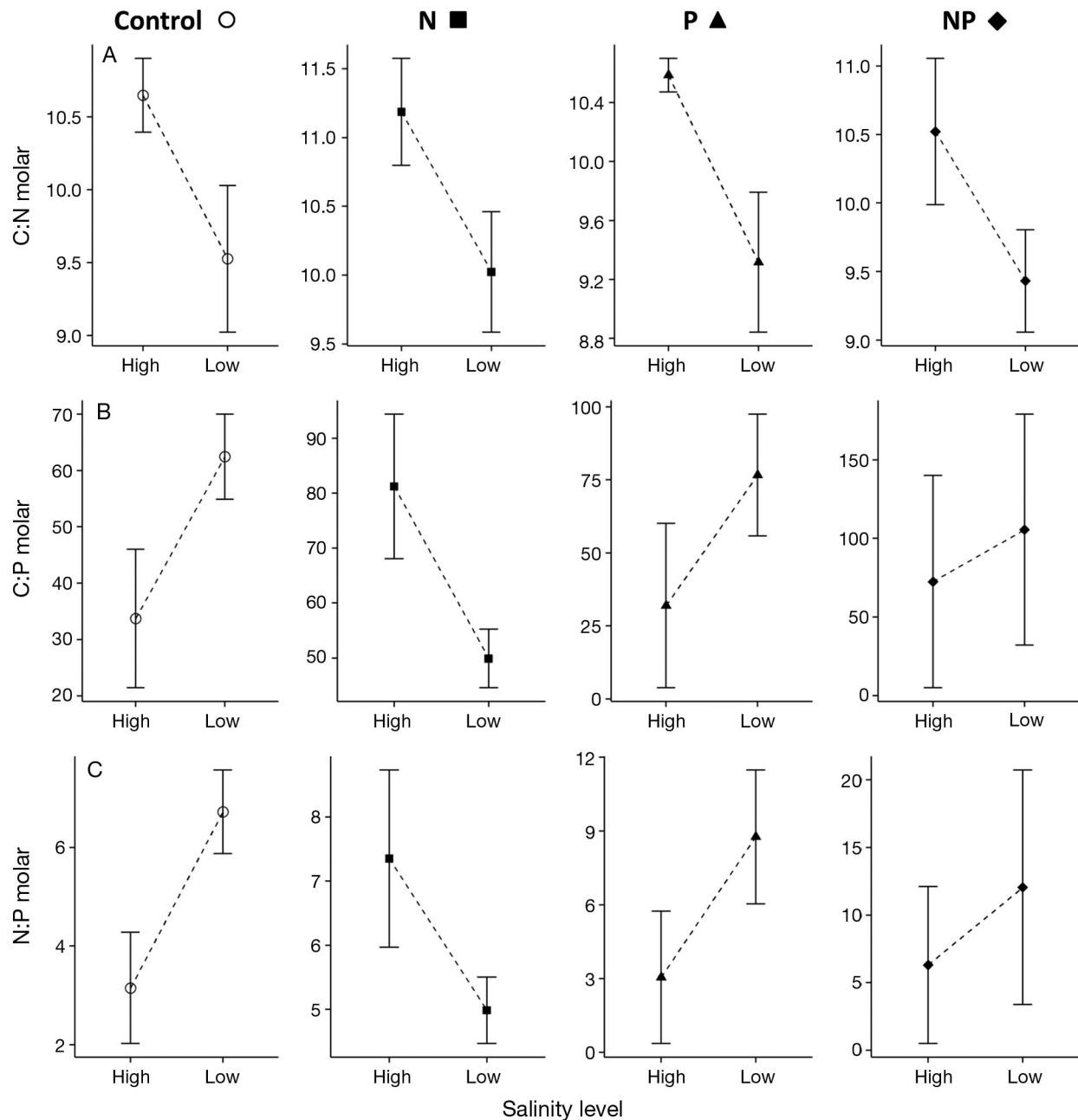


Fig. 4. Stoichiometric molar ratios of (A) carbon:nitrogen (C:N), (B) carbon:phosphorus (C:P) and (C) nitrogen:phosphorus (N:P) for each nutrient treatment (nitrogen, N; phosphorus, P; nitrogen + phosphorus, N+P; control) vs. salinity level (high:  $\geq 20$  ppt, low:  $< 5$  ppt). Shapes are mean values, and error bars are  $\pm 1$  standard error

fore already be in excess of what is required by the epilithon community for optimal growth. The strongest support for this comes from unnoticeable increases in chl *a* or AFDM on plates that received nutrient additions, including high salinity pools where background nutrient concentrations were lowest. Further support for a preexisting surplus of background N and P comes from the lack of effects from nutrient additions on epilithon C:N:P ratios (Fig. 4).

Similar nutrient enrichment treatments in coastal and marine ecosystems have resulted in increases in epilithon and autotrophic biomass, but these effects may have been attributed to additions to considerably lower background nutrient concentrations, which were consistently  $< 2.0 \mu\text{M NO}_3^-$  and  $< 0.8 \mu\text{M SRP}$  (Hillebrand et al. 2000, Hillebrand & Kahlert 2001, Smith et al. 2001). Our results are also distinct from Dalton et al. (2012), who suggested that higher

background nutrient concentrations are advantageous to benthic primary production, which may have been due to their sampling across a different range of background  $\Sigma\text{NO}_3^-$  and salinities. In particular, pools sampled by Dalton et al. (2012) were across a wider range of mean background  $\Sigma\text{NO}_3^-$  concentrations (131.4  $\mu\text{M}$  difference) compared to ours (96.3  $\mu\text{M}$  difference) and were also at the higher end of the background  $\Sigma\text{NO}_3^-$  gradient, ranging from 50.4 to 181.8  $\mu\text{M}$ , with salinities consistently <7 ppt. On the other hand, while we sampled across a lower and smaller range of background  $\Sigma\text{NO}_3^-$ , our pools ranged more in salinity between 2.3 and 22.0 ppt. Therefore, the effects of nutrient addition on epilithon communities may be influenced by co-varying factors such as salinity and may explain our dissimilar results to Dalton et al. (2012).

Also in contrast to our first hypothesis was the negative relationship between groundwater influence and both AFDM and chl *a*. Negative relationships between nutrient loads and primary production in certain estuaries and lakes have been attributed to limitation by light, temperature and toxic contaminants, where nutrient concentrations are high (Yoshizawa & Sharp 2006, Karlsson et al. 2009). However, light was unlikely to be a factor in this study, as the pools sampled were open, with no vegetative cover, and were shallow, and water clarity was extremely high (T. S. Sakihara pers. obs.).

Aside from nutrient availability, salinity may impose limits on autotrophy and epilithon biomass, and both positive and negative relationships have been found between benthic microalgal production and salinity in estuaries (Blasutto et al. 2005, Alberti et al. 2010). Inverse relationships of benthic microalgal production and salinity have previously been described in lagoons, but greater benthic microalgal production and lower diversity in more saline and stable conditions have also been found (Blasutto et al. 2005). In our study, autotrophy and epilithon biomass were greater in pools with higher salinity and less groundwater influence. However, it is interesting to note that our ungrazed treatments in low salinity and higher background  $\Sigma\text{NO}_3^-$  pools showed a doubling of chl *a* concentrations with N and N+P additions, whereas chl *a* was seemingly suppressed in N and P treatments in high salinity and lower background  $\Sigma\text{NO}_3^-$ . C:N and N:P ratios on ungrazed treatments were also influenced by salinity, as epilithon from lower salinity pools had higher nitrogen content. Microscopic examination (400 $\times$  magnification) of ungrazed control samples from pools in high salinity consistently showed a dominance of small

unidentified diatoms, while ungrazed control samples from pools in low salinity varied in composition comprised of different functional algal groups, such as small unidentified diatoms, cyanobacterium (*Leptolyngbya* sp.) and filamentous algae (*Stigeoclonium* sp.) (A. R. Sherwood pers. comm.). This suggests that salinity influenced the microalgal community composition, which may have responded differently to nutrient treatments and therefore caused differences in AFDM, chl *a*, AI and ungrazed C:N and N:P ratios between salinity levels. Further, the contrasting responses of ungrazed microalgae to nutrient treatments between salinity levels suggest that the effects of groundwater on epilithon are more involved than nutrients or salinity acting alone.

### Endemic grazer influences on epilithon

Our findings indicate that grazing, particularly by *H. rubra*, significantly decreased autotrophy and epilithon biomass. *H. rubra* made up the vast majority of grazers (>99%), which verifies their pervasiveness across all Hawaiian anchialine pools, as do previous studies (Maciolek & Brock 1974, Chan 1995, Sakihara 2012). However, *H. rubra* are highly cryptic and are capable of migrating freely through hypogeal connections across pools; thus, obtaining density estimates that accurately reflected grazing pressure was exceedingly difficult, and our measurements may be a conservative estimate of their populations. These behaviors likely contributed to the lack of density-mediated effects of *H. rubra* on epilithon biomass, which was expected to have similar predicting accuracy and power to grazer presence or exclusion. The presence of *H. rubra* in treatments alone was shown on average to decrease AFDM by 73 % and chl *a* by 90 % and to increase AI by 665 % and C:N ratios by 24 %.

Visual observations of the pigments of our epilithon samples suggested differences in epilithon composition between grazing treatments, as ungrazed samples were consistently brown, while grazed samples were consistently green. Microscopic examination (400 $\times$  magnification) of green grazed epilithon was comprised of a mixture of filamentous algae (*Stigeoclonium* sp.), small unidentified diatoms and cyanobacterium (*Leptolyngbya* sp.), whereas brown ungrazed samples were less diverse and comprised mainly of small unidentified diatoms (A. R. Sherwood pers. comm.). These qualitative differences begin to suggest that *H. rubra* selectively graze on epilithon, supported by the fact that epilithon compositions in grazed samples were more heterotrophic than un-

grazed samples and that C:N ratios were consistently greater in grazed samples. However, supplementary investigations of the epilithon community structure must be conducted to confirm this selective grazing behavior and apparent differences in algal functional groups. Together, these findings support our second hypothesis that *H. rubra* controls epilithon biomass and structure through grazing.

The magnitude of response by the epilithon community to grazing may be a function of differences in epilithon assemblage between contrasting salinity levels and suspected selective grazing behavior of *H. rubra*. In this study, decreases in autotrophic biomass (increased AI) from grazing were seemingly amplified in pools with lower salinity and more groundwater influence (Fig. 3C), although this did not result in a significant ANOVA interaction effect. In low salinity pools, lower concentrations of chl *a* and AFDM indicated less availability of food, particularly the autotrophic component of epilithon. These smaller proportions of autotrophs would result in a greater percentage consumed by *H. rubra* relative to the total amount of autotrophic material available and result in a greater response of AI to grazing. Yet, subsequent investigations of the taxonomic assemblages of the epilithon food web structure using stable isotope techniques and density-mediated grazer effects across a salinity/groundwater gradient are warranted to better describe the intricacies of *H. rubra* grazing effects.

The functional traits of *H. rubra* and their controls on benthic autotrophy and epilithon demonstrated in this study further validate its keystone status in Hawaiian anchialine pools. Likewise, our results support the predication that any mechanism that alters *H. rubra* grazing pressure will drive significant changes in benthic autotrophy and epilithon structure, such as the presence of invasive alien fish (e.g. poeciliids and tilapia). Reduced grazing frequency and altered behavior of *H. rubra* due to the presence of these fishes is likely to contribute to shifts in epilithon community structure, such as lowered diversity (Lowe & Hunter 1988, Bailey-Brock & Brock 1993), increased standing biomass (Dalton et al. 2012) and a potential ecological shift primarily through non-consumptive effects (Havird et al. 2013). The susceptibility of *H. rubra* to invasive fishes and commensurate trophic cascades in anchialine pools is testament to the particular vulnerability of island ecosystems (Reaser et al. 2007) and a prime example of the degrading effects of some of the most globally invasive aquatic species on native ecosystems (Lowe et al. 2000, MacKenzie & Bruland 2012, Holitzki et al. 2013).

## CONCLUSION

Hawaiian anchialine pools are distinct from those of many other tropical coastal ecosystems, because of their unique biota, hydrology and physical characteristics. In this instance, they have provided insight into lower-level trophic interactions over a range of dynamic yet consistently eutrophic conditions. The pools allowed us to demonstrate that epilithon biomass and structure are not discernibly nutrient limited in some Hawaiian tropical coastal systems and may rather be influenced by a complexity of factors associated with groundwater. This suggests that a finer interplay among salinity, background nutrients and perhaps other environmental factors is driving bottom-up control of primary production.

The functional role displayed by *Halocaridina rubra* in anchialine pools in this study underscores the importance of grazers in tropical nearshore waters and the need for healthy populations of these engineering organisms to maintain the integrity of aquatic habitats. Migratory restraints of *H. rubra* exist across island subregions caused by geological and hydrological characteristics, thus creating seemingly allopatric populations of *H. rubra* (Santos 2006). Consequently, refuge from degraded habitat would seem to be limited as well. In efforts to conserve these delicate ecosystems and their endemic species, controlling local and regional impacts to water quality (i.e. anthropogenic pollutants, groundwater removal) and the physical habitat (i.e. development over and immediately around existing pools) that may alter grazer abundance or behavior must therefore be considered. Furthermore, given that Hawaiian anchialine pools are geologically ephemeral systems, we suggest a longer-term study similar to our design that investigates grazing effects on microalgal growth on natural substrata in the pools over potential shifts in background habitat conditions. This would advance our understanding of these interactions over the geological lifespan of these pools.

In light of the rarity and delicate nature of Hawaiian anchialine habitats, mitigating introductions of invasive fishes and plants is a foremost priority if these habitats are to be preserved, especially as they support a highly endemic assemblage of shrimp and other invertebrates (Nature Conservancy 1987, Chan 1995, Brock 2004). We infer that preserving or restoring intact native grazer communities such as those found in Hawaiian anchialine pools through increased public awareness, protecting pristine habitats and initiating intensive removal efforts of invasive fishes and plants is central towards maintaining



the resiliency of these tropical coastal ecosystems to biotic and abiotic impacts. Such actions may aid in preventing anchialine ecosystem shifts to degrading alternative stable states (Scheffer et al. 2001). This is particularly crucial amid increasing anthropogenic influences across tropical coastal ecosystems that depend primarily on both freshwater discharge and the ocean.

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**Appendix.** Results of 1-way (Table A1), 3-factor (Table A2) and 2-factor (Table A3) analysis of variance (ANOVA)

Table A1. One-way ANOVA results of epilithon response variables, chlorophyll *a* (chl *a*,  $\mu\text{g cm}^{-2}$ ), ash-free dry mass (AFDM,  $\mu\text{g cm}^{-2}$ ), autotrophic index (AI, AFDM chl  $\text{a}^{-1}$ ) and carbon:nitrogen (C:N) molar ratios between procedural control and grazed control treatments

Response	Source	df	SS	MS	<i>F</i>	<i>p</i>
Chl <i>a</i>	Control treatments	1	0.27	0.27	0.08	0.79
	Residuals	20	70.3	3.50		
AFDM	Control treatments	1	0.09	0.09	2.09	0.16
	Residuals	20	0.84	0.04		
AI	Control treatments	1	0.0004	0.0004	1.25	0.28
	Residuals	20	0.0068	0.0003		
C:N	Control treatments	1	0.57	0.57	3.30	0.08
	Residuals	20	3.46	0.17		

Table A2. Three-factor ANOVA results of grazing treatment (GT), nutrient treatment (NT), salinity (S) and their interactions on epilithon response variables chlorophyll *a* (chl *a*,  $\mu\text{g cm}^{-2}$ ), ash-free dry mass (AFDM,  $\mu\text{g cm}^{-2}$ ), autotrophic index (AI, AFDM chl  $a^{-1}$ ) and carbon:nitrogen (C:N) molar ratios. Significant results (in **bold**) are reported on false discovery rate-adjusted p-values  $\leq 0.05$  (Benjamini & Hochberg 1995) and type II sums of squares

Source	df	SS	MS	<i>F</i>	p
Chl <i>a</i>					
GT	1	843.5	843.5	179.03	<b>&lt;0.001</b>
NT	3	29.2	9.7	2.07	0.157
S	1	322.2	322.2	68.38	<b>&lt;0.001</b>
GT:NT	3	4.6	1.5	0.33	0.807
GT:S	1	15.0	15.0	3.19	0.157
NT:S	3	31.4	10.5	2.22	0.157
GT:NT:S	3	6.1	2.0	0.43	0.807
Residuals	72	339.2	4.7		
AFDM					
GT	1	794.3	794.3	139.65	<b>&lt;0.001</b>
NT	3	8.9	3.0	0.52	0.780
S	1	450.3	450.2	79.16	<b>&lt;0.001</b>
GT:NT	3	3.2	1.1	0.19	0.904
GT:S	1	20.2	20.2	3.55	0.148
NT:S	3	29.3	9.8	1.72	0.239
GT:NT:S	3	38.9	13.0	2.28	0.151
Residuals	72	409.5	5.7		
AI (AFDM chl $a^{-1}$ )					
GT	1	27.5	27.54	89.89	<b>&lt;0.001</b>
NT	3	2.8	0.94	3.06	0.079
S	1	6.1	6.06	19.78	<b>&lt;0.001</b>
GT:NT	3	0.7	0.24	0.78	0.554
GT:S	1	1.1	1.12	3.67	0.104
NT:S	3	1.9	0.63	2.04	0.162
GT:NT:S	3	0.6	0.22	0.70	0.554
Residuals	72	22.1	0.31		
C:N					
GT	1	0.00141	0.00140	18.43	<b>&lt;0.001</b>
NT	3	0.00052	0.00017	2.25	0.208
S	1	0.00031	0.00031	4.12	0.161
GT:NT	3	0.00035	0.00012	1.51	0.383
GT:S	1	0.00006	0.00006	0.77	0.538
NT:S	3	0.00006	0.00002	0.28	0.885
GT:NT:S	3	0.00005	0.00002	0.22	0.885
Residuals	72	0.00549	0.00008		

Table A3. Two-factor ANOVA results of nutrient treatment (NT) and salinity (S) and their interactions on epilithon response variables carbon:nitrogen (C:N), carbon:phosphorus (C:P) and nitrogen:phosphorus (N:P) molar ratios. Significant results (in **bold**) are reported on false discovery rate-adjusted p-values  $\leq 0.05$  (Benjamini & Hochberg 1995) and type II sums of squares

Source	df	SS	MS	<i>F</i>	p
C:N					
NT	3	3.1	1.0	0.78	0.77
S	1	11.8	11.8	8.75	<b>0.02</b>
NT:S	3	0.04	0.0	0.01	1.00
Residuals	36	48.3	1.3		
C:P					
NT	3	2.8	0.9	0.43	0.73
S	1	9.5	9.5	4.41	0.13
NT:S	3	12.0	4.0	1.86	0.23
Residuals	36	77.9	2.2		
N:P					
NT	3	1.5	0.5	0.44	0.73
S	1	7.7	7.7	6.69	<b>0.04</b>
NT:S	3	6.8	2.3	1.98	0.20
Residuals	36	41.5	1.2		

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