

**Endemic Grazer (*Halocaridina rubra*) Forces and Obscured Nutrient Effects on
Benthic Autotrophy in Hawaiian Anchialine Pools**

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Abstract

Anthropogenic impacts on aquatic ecosystems alter the controls of epilithon structure and biomass by altering top-down and bottom-up forces, and their interactions. In Hawai‘i, these impacts include increased nutrient loading from development and invasive nitrogen-fixing plants, as well as the continual spread of other alien invasive species. This study examined the effects of nutrient availability and the grazing effects of an endemic aytid shrimp, *Halocaridina rubra*, on epilithon structure and biomass in Hawaiian anchialine pools. Epilithon was sampled by deploying terra-cotta plates with a combination of nutrient enrichment and grazer exclusion treatments in anchialine pools across a background $\text{NO}_2^- + \text{NO}_3^-$ concentration gradient. Autotrophy and epilithon biomass significantly decreased across this background $\text{NO}_2^- + \text{NO}_3^-$ gradient and with grazing by *H. rubra*, whereas the effect of grazing treatments on relative autotrophy (ash-free dry mass chlorophyll a^{-1}) was more pronounced in pools with higher background $\text{NO}_2^- + \text{NO}_3^-$ concentrations. However, minimal effects of nutrient enrichment treatment were found and no algal communities from pools sampled appeared to be nutrient limited over the range of background $\text{NO}_2^- + \text{NO}_3^-$ concentrations investigated. Relatively high background $\text{NO}_2^- + \text{NO}_3^-$ concentrations in the pools may be responsible for the lack of nutrient treatment effects. Instead, potential effects of salinity and nutrient toxicity on epilithon may be important factors. My results suggest that grazing by *H. rubra* is a primary driver of epilithon structure and biomass and that this endemic grazer is a key species in maintaining the natural conditions of Hawaiian anchialine pools across a range of nutrient concentrations and abiotic conditions.

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Introduction

Coastal ecosystems worldwide are experiencing severe ecological impacts, such as loss of biodiversity, shifts in species composition, and altered ecosystem function due to nutrient inputs from population increase, urbanization, and the spread of nitrogen-fixing plants (Vitousek et al. 1997, McClelland and Valiela 1998). For instance, coastal regions along the western side of Hawai‘i Island have experienced habitat destruction from rapid development with consequent increases in nutrient inputs from sewage and fertilizer use (Wiegner et al. 2006, Bruland and MacKenzie 2010). Introduced invasive plant and fish species along Hawai‘i’s coasts have increased as a result of human population growth and development (Bantilan-Smith et al. 2009, MacKenzie and Bruland 2011). Because these delicate native communities are condensed over the small and isolated Hawaiian archipelago, the effect of various biotic and abiotic stressors acting upon them can be considerably augmented (Paulay 1994, Reaser et al. 2007). Of special interest and concern are the anchialine pools and their constituent endemic species along the impacted coastal regions of Hawai‘i.

Anchialine pools are mixohaline environments that exhibit dampened tidal fluctuations with no surface connection to the sea (Holthuis 1973). The global majority of these unique environments are located along the coastline of the main Hawaiian Islands, of which > 70% are on the western coast of Hawai‘i Island (Brock et al. 1987). Typically, these pools are located in relatively barren basaltic lava fields with uneven basins and are usually < 0.5 m deep. Hawaiian anchialine pools are also considered some of the most biologically diverse anchialine ecosystems throughout the Indo-Pacific (Maciolek 1986), supporting unique assemblages of aquatic animals, algae and microbes (Brock 2008).

The predominant faunal species in Hawaiian anchialine pools is an endemic atyid shrimp, *Halocaridina rubra*, a benthic microphagous grazer that feeds on autotrophic and detrital material (Wong 1975, Maciolek 1983, Bailey-Brock and Brock 1993). Due to its ubiquity, relative lack of native competitors, and high densities, *H. rubra* is considered a keystone species to Hawaiian anchialine ecosystems by serving an instrumental role in regulating its epilithon biomass and community structure (Bailey-Brock and Brock 1993, Capps et al. 2009, Dalton et al. 2012). The unique flora of Hawaiian anchialine pools, comprised of at least 144 recorded species of macroalgae, diatoms and microalgae (Wong 1975), is thereby suggested to be influenced by the presence and behavior of *H. rubra*.

The unique physical, hydrological, and ecological characteristics of anchialine pools in Hawai'i make them inherently sensitive to both biotic and abiotic stressors. For instance, significant decreases in abundance, diurnal extirpation and altered behavior of *H. rubra* caused by the presence of alien, invasive fish, result in shifts in epilithon characteristics and food web dynamics (Bailey-Brock and Brock 1993, Capps et al. 2009, Carey et al. 2010, Dalton et al. 2012). Also, increases in nutrient loads in groundwater, which have been extensively examined along the western coast of Hawai'i Island, may be detrimental to anchialine pools (Brock et al. 1987, Brock and Kam 1997, Wiegner et al. 2006, Bruland and MacKenzie 2010, Knee et al. 2010). Such increases in nutrient concentrations cause significant increases in standing autotrophic biomass (Dillon and Rigler 1974) and primary production in aquatic ecosystems (Nixon et al. 1996).

Interactive effects of top-down and bottom-up forcing on epilithon structure and biomass, rather than their independent effects, have become an increasing focus of investigation in various

aquatic ecosystems (Rosemond et al. 1993, Smith et al. 2001, Hillebrand and Kahlert 2001, Alberti et al. 2010). Increases in epilithon biomass due to nutrient enrichment may be followed by increased grazer density, growth, or migration to nutrient-enriched- and therefore food-enriched- sites (Rosemond et al. 1993, Nisbet et al. 1997, Hillebrand et al. 2000, Roll et al. 2005). Alternatively, primary grazers may respond negatively 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 and Fong 2004).

In Hawaiian anchialine pools, Dalton et al. (2012) have described concurring grazer density and trait-mediated effects (i.e., altered diel migratory behavior) together with nutrient loads, as epilithon biomass and composition were controlled by both bottom-up nutrient loading and top-down fish presence, which reduced *H. rubra* abundance and altered their grazing behavior. Such evidence suggests that Hawaiian anchialine ecosystems respond similarly to stressors as other aquatic ecosystems. However, nutrient concentrations tend to vary predictably across a salinity gradient (Street et al. 2008, Knee et al. 2010), and the effects of nitrogen and phosphorus enrichment on anchialine pools along this existing gradient have yet to be experimentally examined. Furthermore, the total density dependent effect of grazing by *H. rubra* (i.e., relative to an absence of grazing) on epilithon structure and biomass has never been quantified through manipulation of *H. rubra* densities in Hawaiian anchialine pools.

This study examined the independent and combined effects of grazing pressure and nutrient availability on epilithon structure and biomass in Hawaiian anchialine pools across a nutrient concentration gradient. My goals were to identify the impacts of 1) increased concentrations of nitrogen, phosphorus, or a combination of these two nutrients, 2) exclusion of

total grazing by *H. rubra*, and 3) the concomitant effects of these two factors on epilithon structure and biomass in Hawaiian anchialine pools across a nutrient concentration gradient. I predicted that autotrophy (autotrophic biomass) and epilithon biomass would be significantly altered with nutrient enrichment treatments, particularly in lower background nutrient conditions indicating nutrient limitation. I also predicted that epilithon biomass would be significantly affected by grazing by *H. rubra*. Lastly, I predicted that the combination of increased nutrient loading and grazing would have no effect on epilithon structure and biomass, presuming their independent effects are contrasting. My objectives were to advance the understanding of anchialine ecosystem response to the combined effects of specific biotic and abiotic stressors, and to provide further understanding of Hawaiian anchialine ecosystems for improved management of these highly impacted, unique ecosystems.

Methods

Study Sites and Habitat Characteristics

Surveys and sampling were conducted in 11 anchialine pools located at Kapalaoa, 'Akahukaimu, Weliweli and Manukā on the western coast of Hawai'i Island from September 30, 2011 to October 28, 2011 (Figure 1). Due to the perpetual northeasterly trade winds and orographic rainfall along the eastern side of the island, a consequent rain shadow on the western coast results in drier climate and less rainfall within this region. At the southernmost site at Manukā, three 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 25,550 acres of undeveloped land. Sampled pools were selected based on four criteria: 1) lack of canopy vegetation, 2) lack of introduced or native fish, 3) an established population of *H. rubra*, and 4) background nutrient concentrations that spanned across a naturally occurring gradient. Aside from small stands of alien *Prosopis pallida*, all pools generally lacked peripheral vegetation.

Measurements of physiochemical parameters and habitat characteristics of each sampled pool were conducted immediately prior to placement of experimental arrays. Measurements included: water temperature (°C), salinity (ppt), conductivity (mS cm⁻¹), pH, dissolved oxygen (mg l⁻¹), turbidity (NTU), water depth (cm), pool surface area (m²), and pool type (single pool or pool complex).

Experimental Design and Methodology

Microalgae were sampled using 8-cm diameter unglazed terra-cotta plates, which were used as algae recruitment tiles (Pringle and Triska 2006). Nine plates (an experimental array) were deployed in each pool for 29 days to concurrently examine the effects of two 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 four nutrient enrichment treatments: nitrogen (N), phosphorus (P), nitrogen+phosphorus (N+P), and a control with no nutrient enrichment for a total of eight plates. Nutrient enrichment was accomplished by using diffusion media composed of a 3% agar solution that was either 5 M NaNO_3 for N treatments, 1 M KH_2PO_4 for P treatments, and 5 M NaNO_3 + 1 M KH_2PO_4 for N+P treatments (Tank et al. 2006). Control treatments were comprised of only 3% agar solution. Sixty 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 surface 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 μm Nitex® bolting cloth glued to a 10 cm diameter plastic petri dish frame. Plates open to grazers were similarly constructed, except with four openings (3.2 cm^2) on the sides of the frame to allow grazers onto the diffusing surface. The remaining 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 in areas of the pools that were most exposed to sunlight and were constantly submerged under water. 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 two hours after deployment and immediately before the plates were removed (29 days).

Additionally, water samples were collected from each pool approximately 2-3 m away from the plates to measure background $\text{NO}_2^- + \text{NO}_3^-$, soluble reactive phosphorus (SRP) and NH_4^+ concentrations. $\text{NO}_2^- + \text{NO}_3^-$ 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 μm (GF/F) filters and analyzed for $\text{NO}_2^- + \text{NO}_3^-$ (detection limit (DL): 0.1 μM , USEPA 353.4) and SRP (DT: 0.1 μM , USEPA 365.5) using a Pulse Technicon II™ autoanalyzer.

Visual Surveys of Grazers

Densities of *H. rubra* and other benthic grazers, as well as species composition of all benthic invertebrates were estimated by a visual sampling method using quadrats. Each pool was systematically surveyed along a transect, which was formed by measuring the longest length across the pool using a metric tape. A minimum of four visual quadrat (0.06 m²) samples were conducted equidistantly along the transect. The quadrat was placed on the bottom and left undisturbed for one minute, after which visual counts of all benthic motile animals located within the quadrat area were recorded. These data provided verification of the presence of *H. rubra* and calculated density averages 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. 2010, Sakihara 2012). Therefore, in order to ensure consistent estimates of *H. rubra* density, visual sampling was conducted during the day and night within the same tidal cycle and tidal period (two hours within low tide).

Sample Processing and Laboratory Analysis

After 29 days of deployment, all algal samplers were removed from the pools and processed in the field. The flat 53.5 cm² diffusing surface of each sampler was gently scrubbed clean with a stiff bristled brush for one minute and rinsed into a slurry of known volume (ml) with water from the sampled pool that had been filtered through a 0.7 µm GF/F filter. Slurry samples were stored in the dark and chilled (4 °C; Pringle and Triska 2006). Subsamples of the aliquot were then vacuum-filtered at the University of Hawai'i at Hilo Analytical Laboratory through precombusted 0.7 µm GF/F filters. Separate subsamples were processed for analyses of chlorophyll *a* (Chl *a*), ash-free dry mass (AFDM), and particulate C, N, P (Hillebrand and Kahlert 2001).

Epilithon Chl *a* content, a measure of autotrophic biomass, was quantified using a Turner Designs™ (model 10-AU) fluorometer (Arar and Collins 1997). Chl *a* was extracted from the filtered samples in 5 ml of 90% buffered acetone at -15 °C for 24 hours. Initial fluorometric readings were recorded, then each sample was acidified by adding three drops of 0.1 N HCl and allowed to steep for one minute. 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, were filtered on pre-weighed 0.7 µm GF/F filters and then dried for 48 hours at 60 °C. The filters were then weighed on an analytical microbalance (to the nearest 1.0 µg), ashed at 500°C for four hours and reweighed. AFDM concentration was then calculated by methods described by Steinmen et al. (2006). An autotrophic index, AFDM / Chl *a* (AI), 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 hours at 60 °C, rolled in tin 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 and Fanta (2008). The P samples were analyzed on a Varian Vista MPX ICP-OES spectrometer. Particulate P was only detectable in ungrazed treatment samples, and therefore was not analyzed on 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 $\text{NO}_2^- + \text{NO}_3^-$ and SRP concentrations of algal plate surface samples between nutrient treatments followed by a *post-hoc* Tukey's HSD test to verify effective N and P diffusion for desired treatments. One-way ANOVA tests were also used to compare AFDM, Chl *a*, AI and C:N between procedural control treatments and control treatments to address potential confounding effects of grazer exclusion hardware or agar media. In addition, one-way ANOVA tests were used to compare diel 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 one-way ANOVA test. Spearman's rank correlations were also used to examine relationships between physiochemical parameters and background nutrient conditions and *H. rubra* densities.

Generalized linear mixed models (GLMM) with residual maximum likelihood estimations were used to examine the effects of nutrient additions and grazing treatments on

AFDM, Chl *a*, AI, and C:N while accounting for restricted randomization, and treating background nutrient concentrations as a covariate. Nutrient concentrations have been inversely correlated to salinity in estuarine habitats (Rudek et al. 1991). This was verified through significant correlations between salinity and background $\text{NO}_2^- + \text{NO}_3^-$ concentrations ($r = -0.88$, $p < 0.001$) from anchialine pool surveys conducted in October and November 2010. Also, conductivity is a function of salinity, and pH has been shown to increase with salinity, particularly at lower levels (Wong 1979). Therefore, in order to avoid multicollinearity, salinity, conductivity and pH were not included in the statistical models, but was addressed through nutrient addition treatments. All response variables were log-transformed and different residual variances were fitted to the two grazing treatments to improve the fit of the models. Significant interactions of predictor variables were followed by an analysis of simple effects and Tukey's HSD test.

GLMM tests were also used to explicitly examine the separate effects and interactions of nutrient treatment and background nutrients on C:N, C:P and N:P molar ratios of ungrazed treatments. Again, background nutrients were treated as a covariate and all response variables were natural log-transformed to improve model fits. In addition, different residual variances were fitted to the nutrient treatments to improve the fit of the models.

Best-fit models were determined by using the Corrected Akaike Information Criterion (AICc), with lower scores indicating better fit (Akaike 1974). ANOVA and Tukey's HSD tests were conducted using R statistical software (R Development Core Team, 2010), and GLMM tests and analysis of simple effects were done in PROC mixed using SAS software (SAS Institute Inc., Cary, NC, USA).

Results

Habitat Characteristics and Grazer Density

Pool characteristics varied considerably among sampling sites. Background $\text{NO}_2^- + \text{NO}_3^-$ concentrations ranged from $< 10 \mu\text{M}$ to $> 80 \mu\text{M}$ between pools (Table 1) and was correlated with pH ($\rho = -0.97$, $p < 0.001$). Pool surface area ranged from a 1 m^2 pool complex to a 228 m^2 single pool (Table 2). Salinity ranged from 2.3-22.0 ppt (Table 2). However, as pH has been shown to increase with salinity, particularly at lower levels (Wong 1979), and because conductivity is a function of salinity, these parameters were not included in the statistical models. Aside from correlations across salinity, pH and background nutrients, no other correlations were observed between physical parameters and background nutrient concentrations or *H. rubra* densities.

Average *H. rubra* densities (\pm SD) ranged from $167 \pm 6.6 \text{ no m}^{-2}$ to $1800 \pm 59.7 \text{ no m}^{-2}$ across all pools (Table 2). Several pools also contained other less common benthic invertebrates, such as *Metabetaeus lohena* and amphipods. Nonetheless, *H. rubra* was the dominant species present in all pools and no differences in average densities were detected between day and night surveys. Based on the cryptic behavior of *H. rubra*, the rugosity of the pool basins, and the consequent high degree of variability in density estimates, 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 $\text{NO}_2^- + \text{NO}_3^-$ (μM) among the four nutrient treatments were significantly different at the start of the experiment (day 1, all stations pooled; ANOVA; $F_{(3, 95)} = 27.727$, $p <$

0.001). Concentrations of $\text{NO}_2^- + \text{NO}_3^-$ were significantly higher in N and N+P treatments (Tukey's HSD, $p < 0.001$), whereas $\text{NO}_2^- + \text{NO}_3^-$ in P and control treatments were not significantly different from background levels. Analysis of water samples at the end of the experiment (day 29) were consistent with the initial results ($F_{(3, 95)} = 11.253$, $p < 0.001$) with significantly higher $\text{NO}_2^- + \text{NO}_3^-$ in N and N+P treatments compared to background concentrations (Tukey's HSD, $p < 0.01$). As observed for N treatments, SRP concentrations were significantly higher in P and N+P treatments (Tukey's HSD, day 1: $p < 0.001$, day 29: $p < 0.001$), whereas, SRP in N and control treatments were not significantly different from background levels.

No differences were found between procedural control and control treatments for all response variables. The 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 as to keep the designs completely balanced.

Nutrient and Grazing Effects on Epilithon

Interactions of grazing treatment and background nutrient concentrations on AFDM (GLMM; $F_{(1, 36)} = 12.55$, $p < 0.01$) and Chl *a* (GLMM; $F_{(1, 36)} = 12.70$, $p < 0.01$) were shown, as differences between grazing treatments were more pronounced at lower background $\text{NO}_2^- + \text{NO}_3^-$ concentrations (Figure 2a-b). Average AFDM (\pm SE) values were significantly and nearly 4X greater on ungrazed ($1044.5 \pm 161.8 \mu\text{g cm}^{-2}$) compared to grazed treatments ($281.4 \pm 48.7 \mu\text{g cm}^{-2}$) and decreased by $18.5 \mu\text{g cm}^{-2}$ for every $1 \mu\text{M}$ increase in background $\text{NO}_2^- + \text{NO}_3^-$ concentration. On grazed treatments, AFDM (\pm SE) decreased by $1.9 \mu\text{g cm}^{-2}$ for every $1 \mu\text{M}$

increase in background $\text{NO}_2^- + \text{NO}_3^-$ concentration. Similarly, average Chl *a* (\pm SE) concentrations were significantly and 10X greater on ungrazed ($474.5 \pm 105 \mu\text{g cm}^{-2}$) than grazed treatments ($47.7 \pm 10.9 \mu\text{g cm}^{-2}$), decreasing by $11.6 \mu\text{g cm}^{-2}$ for every $1 \mu\text{M}$ increase in background $\text{NO}_2^- + \text{NO}_3^-$ concentrations. Chl *a* on grazed treatments decreased by $1.8 \mu\text{g cm}^{-2}$ for every $1 \mu\text{M}$ increase in background $\text{NO}_2^- + \text{NO}_3^-$ concentration. No main effects or interactions of nutrient treatments on AFDM and Chl *a* were found (Figure 3a-b).

An interaction between grazing treatment and background nutrient concentrations on AI (GLMM; $F_{(1, 36)} = 6.49, p < 0.05$) was shown, as differences between grazing treatments were more pronounced at higher background $\text{NO}_2^- + \text{NO}_3^-$ concentrations (Figure 2c). However, AI (\pm SE) was instead significantly and nearly 7X lower for ungrazed (4.0 ± 0.5) compared to grazed treatments (26.6 ± 4.5), and increased by 0.018 for every $1 \mu\text{M}$ increase in background $\text{NO}_2^- + \text{NO}_3^-$ concentrations. AI for grazed treatments increased by 0.44 for every $1 \mu\text{M}$ increase in background nutrient concentrations. While there were no main effects or interactions of nutrient treatments on AI, an analysis of simple effects revealed an effect of nutrient treatment in ungrazed treatments ($F_{(3, 30)} = 4.03, p < 0.05$) with higher AI in ungrazed P treatments compared to ungrazed N+P treatments (Tukey's HSD, $p < 0.05$, Figure 3c).

No main effects or interaction of grazing or nutrient treatments was shown for epilithon C:N. Analysis of simple effects revealed significantly greater C:N on grazed versus ungrazed epilithon samples in N treatments ($F_{(1, 36)} = 8.06, p < 0.01$), N+P treatments ($F_{(1, 36)} = 7.63, p < 0.01$) and P treatments ($F_{(1, 36)} = 5.84, p < 0.05$), but not on control treatments. A generally greater C:N ratio (\pm SE) was measured in grazed treatments, averaging 13.1 ± 0.9 , while

ungrazed treatments averaged 9.9 ± 0.3 (Figure 2d). Also, no effects of nutrient treatments or background $\text{NO}_2^- + \text{NO}_3^-$ on C:N were observed independently from grazing (Figure 3d).

C:N:P Stoichiometry

No significant effects of nutrient treatments or background $\text{NO}_2^- + \text{NO}_3^-$ concentrations on C:N or C:P molar ratios was shown (Figures 4a-b). The present lack of any significant effects of nutrient treatment or background nutrients on C:N:P ratios suggest that the epilithon in the pools examined herein are not nutrient limited. However, a marginal effect of background nutrients on N:P molar ratios was revealed ($F_{(1, 9)} = 4.98$, $p < 0.06$) with a general increase in N:P with increasing background nutrient concentrations (Figure 4c). When these stoichiometric ratios were plotted against published nutrient limitation thresholds for benthic microalgae (Hillebrand and Sommer 1999), the observed C:N results had a large range of values, suggesting that a subset of pools may be N limited (Figure 5a). In contrast, nearly all epilithon samples did not appear to be P limited (Figure 5b).

Discussion

The experimental design and methods used in this study proved to be effective with no confounding effects, thus confirming that the response of epilithon to nutrients and grazing treatments were valid. As in other nutrient enrichment studies (Hillebrand and Kahlert 2001, Tank and Dodds 2003, Scott et al. 2009) the nutrient diffusion media successfully enriched the sampling surface of the terra-cotta plates with the desired nutrients (N, P and N+P), which was consistently more than 2X the background concentrations throughout the duration of the experiment. In addition, over-diffusion of nutrients across treatments and throughout the pool was not observed. Grazer exclusion cages also successfully prevented *H. rubra* from grazing the sampled surface, 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 days. This was also verified by the lack of differences in all response variables between procedural control and control samples.

In this study, nutrient enrichment did not yield significant effects on autotrophy or epilithon biomass, suggesting no nutrient limitation for these anchialine pools, which is in contrast to lakes and streams investigated in previous studies (Fairchild et al. 1985, Rosemond 1993, Rosemond et al. 1993, Hillebrand and Kahlert 2001). Grazing significantly reduced autotrophic and epilithon biomass, consistent with numerous grazer removal studies (Hillebrand 2002). Also, no interaction of concurrent nutrient and grazing treatments was observed that suggested a dual top-down and bottom-up control on epilithon as described in streams and lakes (Rosemond 1993, Rosemond et al. 1993, Hillebrand and Kahlert 2001). Instead, the interaction between background $\text{NO}_2^- + \text{NO}_3^-$ concentrations and grazing by *H. rubra* had the most significant influence on autotrophy and epilithon biomass. Chl *a* and AFDM concentrations were lower on

grazed treatments and were inversely correlated to background $\text{NO}_2^- + \text{NO}_3^-$ concentrations. This is in partial contrast to Dalton et al. (2012) who described similar reductions with grazing, but rather increases in epilithon biomass with higher background nutrient concentrations. Further, epilithon was more heterotrophic (higher AI) on grazed treatments and at increased background $\text{NO}_2^- + \text{NO}_3^-$ concentrations, as opposed to increased heterotrophy (sediment accumulation) with grazer removal in a tropical stream described by De Souza and Moulton (2005).

Endemic Grazer Influences on Epilithon

The effects of grazing by *H. rubra* on autotrophy and epilithon biomass were more pronounced than nutrient treatments, similar to numerous top-down bottom-up control experiments (Hillebrand 2002). Grazing by *H. rubra* significantly decreased autotrophy and epilithon biomass, which was consistent with results by Dalton et al. (2012). These findings support the predication that *H. rubra* maintains anchialine habitat integrity by influencing epilithon structure through grazing (Bailey-Brock and Brock 1993, Capps et al. 2009, Dalton et al. 2012).

Despite the high degree of variability in *H. rubra* densities, the presence of *H. rubra* in treatments alone was shown on average to decrease AFDM by 73%, Chl *a* by 90%, and increase AI by 665%. Over the study period (29 days), grazing pressure by a low density versus a high density of *H. rubra* on a relatively fixed surface area was indistinguishable. However, *H. rubra* are highly cryptic and are capable of migrating freely through hypogeal connections across pools, thus obtaining accurate density estimates that reflect grazing pressure is exceedingly difficult. Therefore, if accurate measures that truly represent grazing pressure are developed and applied, density-mediated effects of *H. rubra* may be better explained.

My results suggest that any influences on *H. rubra* behavior and presence can have a significant effect on benthic autotrophy and epilithon biomass. Any effects on *H. rubra* may therefore result in rapid habitat degradation, especially as this species exclusively fulfills an ecological niche that is conceivably the most vital to maintaining the physical and biological integrity of the anchialine pools. Given these circumstances, one of the primary threats that merit immediate attention is the introduction of alien invasive poeciliids and tilapia. These species of fish alter the behavior and presence of *H. rubra* through predation, which cause shifts in the epilithon structure that affect the function of the entire anchialine pool ecosystem (Bailey-Brock and Brock 1993, Capps et al. 2009, Carey et al. 2010, Dalton et al. 2012). Nonetheless, the influences of grazing and nutrients must be examined in combination to understand the total effect on epilithon structure and biomass.

Variations in environmental conditions and nutrient loads can influence the magnitude of grazing effects, as the influences of these stressors may be dissociated among trophic levels (Alberti et al. 2010). In this context, decreases in autotrophy and epilithon biomass caused by grazing were amplified in higher background nutrient conditions and lower salinity. In addition, a seemingly greater effect of grazing was observed on C:N with nutrient treatments. The fact that epilithon C:N was greater with grazer presence across nutrient treatments suggests that *H. rubra* may selectively graze on specific epilithon constituents. This is supported by the increase in AI as grazing shifted the epilithon structure more towards heterotrophy. Moreover, observations made during epilithon sample collections suggested differences in pigment of the epilithon between grazing treatments, as ungrazed samples were consistently brown, while grazed samples were consistently green. Microscopic examination (400X magnification) of green-colored epilithon was comprised mainly of filamentous algae, possibly *Stigeoclonium* sp. (Sherwood

pers. comm.) whereas brown, ungrazed samples were comprised of smaller unidentified diatoms. However, further investigation of the epilithon community structure must be conducted in order to confirm selective grazing behavior and apparent differences in algal functional groups.

Abiotic Limitations on Epilithon

Despite the lack of nutrient treatment effects on epilithon biomass and autotrophy, these results were not entirely unforeseen for a few reasons. First, when comparing the background $\text{NO}_2^- + \text{NO}_3^-$ concentrations of the pools to the anchialine water quality standard ($4.67 \mu\text{M}$) used by Wiegner et al. (2006), based on measurements by Brock and Kam (1997), it was shown that all concentrations exceeded the standard (Figure 4). Second, as there are currently no established regulations on water quality for Hawai'i's anchialine pools, comparisons to the mean standard $\text{NO}_2^- + \text{NO}_3^-$ concentration for similar estuarine waters ($0.54 \mu\text{M}$, HDOH 2009) also indicated a relatively high concentration of background nutrients across all pools (Figure 4). Third, although similar nutrient enrichment experiments in coastal and marine ecosystems have described positive effects in benthic production, these effects may have been attributed to the considerably lower background nutrient concentrations, which were consistently $< 2.0 \mu\text{M NO}_3^-$ (Hillebrand et al. 2000, Smith et al. 2000, Hillebrand and Kahlert 2001). In contrast, the pools in this study ranged from $6.3\text{-}102.6 \mu\text{M NO}_2^- + \text{NO}_3^-$, which may already be in excess of what is required by the epilithon community for optimal growth. Taken together, these points suggest that N is not limiting. Further support for this comes from the lack of effects on C:N:P (Figure 4 and 5), which also suggests that P is not limiting.

Negative relationships between excessive nutrient loads and primary production in certain estuaries and lakes have been described, suggesting that light, temperature, and perhaps

toxic contaminants are limiting factors instead (Yoshiyama and Sharp 2006, Karlsson et al. 2009). However, light was unlikely to be a factor in this case, as shading effect was controlled by sampling open pools with no vegetative cover. Mean monthly rainfall- and associated cloud cover- within the sampled regions may have influenced epilithon structure or biomass, although these factors were not measured. Pools with greater autotrophic conditions and epilithon biomass were located in Manukā, which receives more than 3X the amount of mean rainfall in October (45 ± 5.8 mm) than pools at Weliweli, 'Akahukaimu and Kapalaoa (12.8 ± 5.0 mm, Giambelluca et al. 2012). If mean rainfall is used as a proxy of cloud cover or relative decreases in sunlight, light would apparently not be considered a limiting factor.

Salinity may also impose limits on autotrophy and epilithon biomass. According to Alberti et al. (2010), the positive effects of nutrients on primary productivity can be suppressed in higher salinity conditions (> 30 ppt). Inverse relationships of benthic microalgal production and salinity have also been described in lagoons (Blasutto et al. 2005). However, Blasutto et al. (2005) has described greater benthic microalgal production and lower diversity in more saline and stable conditions. In our study, autotrophy and epilithon biomass were greater in pools with higher salinity. These considerably higher salinity pools (Table 2), which also had lower background nutrient concentrations, were located along the coast of the Manukā NAR (Figure 1). Consequently, the long-term and expansive protection of the NAR may have prevented increased nutrient inputs attributed to development and caused less variability in groundwater quality, thereby creating conditions more conducive towards autotrophy. Additionally, the rate of nutrient loading is as equally important to the total nutrient load effect on primary production where functional algal groups respond differently depending on the nutrient loading rate (Butzler and Chase 2009). Therefore, shifts in epilithon structure may also be influenced more

by perpetual nutrient loading rather than relative pulses, despite higher concentrations. This may be especially relevant to smaller pools as flushing rates and residence time of nutrients may be faster and shorter respectively.

Elevated nutrient loads may also have had toxic effects on the epilithon as AFDM and Chl *a* were lower at higher background $\text{NO}_2^- + \text{NO}_3^-$ concentrations. Direct toxic effects of increased nutrient loads on eelgrass (*Zostera*) production have been documented at high concentrations of NH_4^+ by inhibiting growth and photosynthetic processes, and stimulating necrosis (Van Katwijk et al. 1997, Brun et al. 2003, Cabaco et al. 2008). Similarly, low to moderate concentrations of NO_3^- (3.5-7.0 μM) have shown toxic effects on eelgrass production, suggested to be caused by nutrient imbalances and physiological stress, and exacerbated by constant nutrient loading (Burkholder et al. 1992). In addition, some macroalgae (i.e., *Eucheuma denticulatum*) have exhibited a seemingly negative growth response to elevated concentrations of NH_3 (Msuya and Neori 2002). It is thus possible that the epilithon in these anchialine pools are experiencing comparable toxic effects of nutrients, particularly by NO_3^- , as its concentrations are frequently high in numerous pools (Wiegner et al. 2006).

Conclusion

Overall, epilithon biomass and structure in these anchialine pools were not nutrient limited, at least within the range of background $\text{NO}_2^- + \text{NO}_3^-$ concentrations and habitat conditions that were examined. Relatively high background $\text{NO}_2^- + \text{NO}_3^-$ concentrations were suspected to provide sufficient nutrients required by the epilithon, thus precluding any effects of nutrient additions. Grazing by *H. rubra*, however, significantly reduced epilithon and autotrophic biomass across a background $\text{NO}_2^- + \text{NO}_3^-$ concentration gradient. Established populations of *H.*

rubra in Hawaiian anchialine pools were thus demonstrated to be a vital component to maintaining the anchialine pools in its natural state across a range of abiotic conditions.

In efforts to conserve these delicate ecosystems and their endemic species, local and regional impacts to water quality and the physical habitat must be considered. Migratory restraints of *H. rubra* exist across island subregions caused by geological and hydrological characteristics, therefore, creating seemingly allopatric populations of *H. rubra* (Santos 2006). Refuge from degraded habitat caused by extensive nutrient loads, introduced species, or habitat destruction would therefore be limited as well. Considering the rarity and delicate nature of Hawaiian anchialine habitats, mitigating nutrient loading with subsequent impacts on water quality, introductions of invasive species, and pool destruction is a foremost priority if these habitats are to be saved, especially as they support a highly endemic assemblage of shrimp and other invertebrates (Nature Conservancy 1987, Chan 1995, Brock 2004).

Despite our current efforts to preserve and protect Hawaiian anchialine ecosystems, degradation and destruction of these increasingly rare ecosystems persist. Ultimately, expanding studies to investigating trophic interactions, primary (and secondary) production and food web structure, will provide a more holistic understanding of Hawaiian anchialine habitats. These dynamic measures of anchialine ecology may reveal greater insight into the effects of excessive nutrient loads and invasive species, which would proportionately improve conservation strategies that can mitigate such effects. But perhaps the most crucial element of effective conservation is understanding what controls, and therefore maintains the resiliency of these anchialine ecosystems from potentially shifting to a degrading alternative stable state (Scheffer et al. 2001). Here I described the grazing mechanism of *H. rubra* that is essential to maintaining the

anchialine habitats in its natural state across a range of nutrient loads. I therefore suggest that examining the physiological resilience of *H. rubra* to organic pollutants, heavy metals, invasive species, and shifts in epilithon structure should be central towards further Hawaiian anchialine studies and establishing a framework for advancing conservation efforts. Finally, the interconnectivity of Hawaiian anchialine habitats with terrestrial and coastal ecosystems also make them naturally sensitive to increased nutrient loads and alien invasive species within these adjoining systems. The anchialine pools may therefore be potentially useful as a model ecosystem to monitor neighboring terrestrial and coastal habitats, deeming them invaluable to multiple ecosystems and the preservation of natural resources of Hawai‘i.

Tables

Table 1. Nutrient concentrations (μM) nitrite+nitrate ($\text{NO}_2^- + \text{NO}_3^-$), soluble reactive phosphorus (SRP), and background ammonium (NH_4^+) at the start and end of the experiment, September 30 to October 28, 2011. Background $\text{NO}_2^- + \text{NO}_3^-$, SRP and NH_4^+ are presented. $\text{NO}_2^- + \text{NO}_3^-$ and SRP are presented for nutrient enrichment treatments nitrogen (N), phosphorus (P) and nitrogen+phosphorus (N+P). ND = no detection.

Site	Pool	Background			Enrichment			
		NO ₂ ⁻ +NO ₃ ⁻	SRP	NH ₄ ⁺	N NO ₂ ⁻ +NO ₃ ⁻	P SRP	N + P NO ₂ ⁻ +NO ₃ ⁻	SRP
START - Day 1								
'Akahukaimu	1	73.61	2.17	1.42	252.92	30.19	239.70	8.19
	2	79.61	1.52	1.02	99.10	6.88	177.43	15.93
	3	73.80	1.90	1.28	283.72	26.14	178.76	19.82
	Mean ± SD	75.68 ± 3.41	1.862 ± 0.33	1.24 ± 0.20	211.91 ± 98.90	21.07 ± 12.46	198.63 ± 35.58	14.65 ± 5.92
Kapalaoa	1	61.21	1.88	*—	390.74	27.65	151.68	12.71
	2	102.64	5.18	1.55	157.47	16.82	223.99	30.68
	3	65.94	1.84	1.74	334.17	58.43	153.33	10.65
	Mean ± SD	76.60 ± 22.68	2.97 ± 1.92	1.65 ± 0.13	294.13 ± 121.68	34.30 ± 21.59	176.34 ± 41.28	18.016 ± 11.02
Manukā	1	11.36	0.19	ND	154.06	45.42	300.67	11.42
	2	6.37	0.00	ND	94.84	51.85	177.34	6.39
	3	8.77	1.18	ND	201.58	120.77	794.28	11.91
	Mean ± SD	8.84 ± 2.50	0.45 ± 0.63	—	150.16 ± 53.48	72.68 ± 41.77	424.10 ± 326.47	9.90 ± 3.06
Weliweli	1	73.85	1.12	0.10	528.68	15.98	153.62	6.88
	2	87.23	1.40	0.82	376.88	39.47	299.78	23.34
		Mean ± SD	80.54 ± 9.47	1.26 ± 0.19	0.46 ± 0.51	452.78 ± 107.34	27.73 ± 16.61	226.70 ± 103.35
END - Day 29								
'Akahukaimu	1	74.45	1.84	—	78.40	4.50	108.61	7.20
	2	67.62	1.58	—	116.43	5.07	81.34	2.32
	3	72.05	1.97	—	98.15	4.15	87.25	3.47
	Mean ± SD	71.37 ± 3.47	1.80 ± 0.20	—	97.66 ± 19.02	4.57 ± 0.46	92.40 ± 14.35	4.33 ± 2.55
Kapalaoa	1	55.40	2.13	—	61.90	9.07	67.30	4.86
	2	39.68	1.71	—	273.75	4.20	471.40	79.87
	3	60.29	2.10	—	417.97	6.68	606.27	180.72
	Mean ± SD	51.79 ± 10.77	1.98 ± 0.23	—	251.21 ± 179.11	6.65 ± 2.43	381.66 ± 280.47	88.48 ± 88.25
Manukā	1	8.35	0.50	—	80.73	2.90	74.57	2.36
	2	10.54	0.56	—	291.38	1.92	254.27	6.21
	3	11.59	1.02	—	57.28	4.71	368.11	6.55
	Mean ± SD	10.16 ± 1.65	0.69 ± 0.29	—	143.13 ± 128.92	3.18 ± 1.42	232.32 ± 148.00	5.04 ± 2.33
Weliweli	1	74.03	1.80	—	101.60	4.37	107.26	2.83
	2	88.85	1.44	—	108.43	5.85	110.87	5.99
		Mean ± SD	81.44 ± 10.48	1.62 ± 0.25	—	105.01 ± 4.83	5.11 ± 1.05	109.06 ± 2.55

*no measurement recorded

Table 2. Physical and physiochemical characteristics, and *Halocaridina rubra* density estimates of each pool.

Site	Pool	Pool Type	Surface Area (m ²)	Maximum depth (m)	Temperature (°C)	Salinity (PSS)	Conductivity (mS cm ⁻¹)	Dissolved O ₂ (mg l ⁻¹)	pH	Mean <i>H. rubra</i> no. m ⁻² ± SD
Akahukaimu	1	single pool	25.1	0.69	25.1	3.9	7.1	8.00	—	167 ± 6.6
	2	single pool	66.2	0.62	25.2	3.1	5.8	8.76	—	400 ± 10.3
	3	single pool	228.0	1.39	25.7	3.8	7.0	8.13	—	283 ± 7.2
	Mean ± SD		106.44 ± 107.26	0.9 ± 0.42	25.33 ± 0.32	3.6 ± 44	6.60 ± 0.72	8.30 ± 0.41	—	275.70 ± 162.33
Kapalaoa	1	complex	1.0	*—	24.5	4.0	7.3	7.75	—	1300 ± 57.8
	2	complex	1.4	0.15	25.9	4.2	7.6	7.69	—	1800 ± 59.7
	3	complex	4.2	0.66	24.4	3.9	7.0	8.16	7.96	283 ± 22.9
	Mean ± SD		2.19 ± 1.72	0.41 ± 0.36	24.94 ± 0.83	4.02 ± 0.17	7.31 ± 0.29	7.87 ± 0.26	8.0	986.11 ± 980.55
Manuka	1	complex	17.5	0.57	24.5	22.0	—	5.88	9.20	1550 ± 82.0
	2	complex	124.6	0.64	24.6	21.0	—	6.33	9.21	283 ± 21.3
	3	complex	46.8	0.55	24.6	20.0	—	6.26	8.74	1233 ± 88.6
	Mean ± SD		62.96 ± 55.32	0.58 ± 0.05	24.58 ± 0.09	21 ± 1.00	—	6.16 ± 0.24	9.05 ± 0.28	1026.39 ± 1253.66
Weliweli	1	single pool	6.4	0.51	23.4	2.8	5.2	5.20	7.96	200 ± 3.6
	2	complex	31.0	0.94	23.9	2.3	4.3	7.99	7.56	267 ± 6.5
	Mean ± SD		18.72 ± 17.4	0.73 ± 0.3	23.66 ± 0.39	2.52 ± 0.38	4.73 ± 0.68	6.60 ± 1.97	7.8	226.04 ± 91.08

*no measurement recorded.

Figures

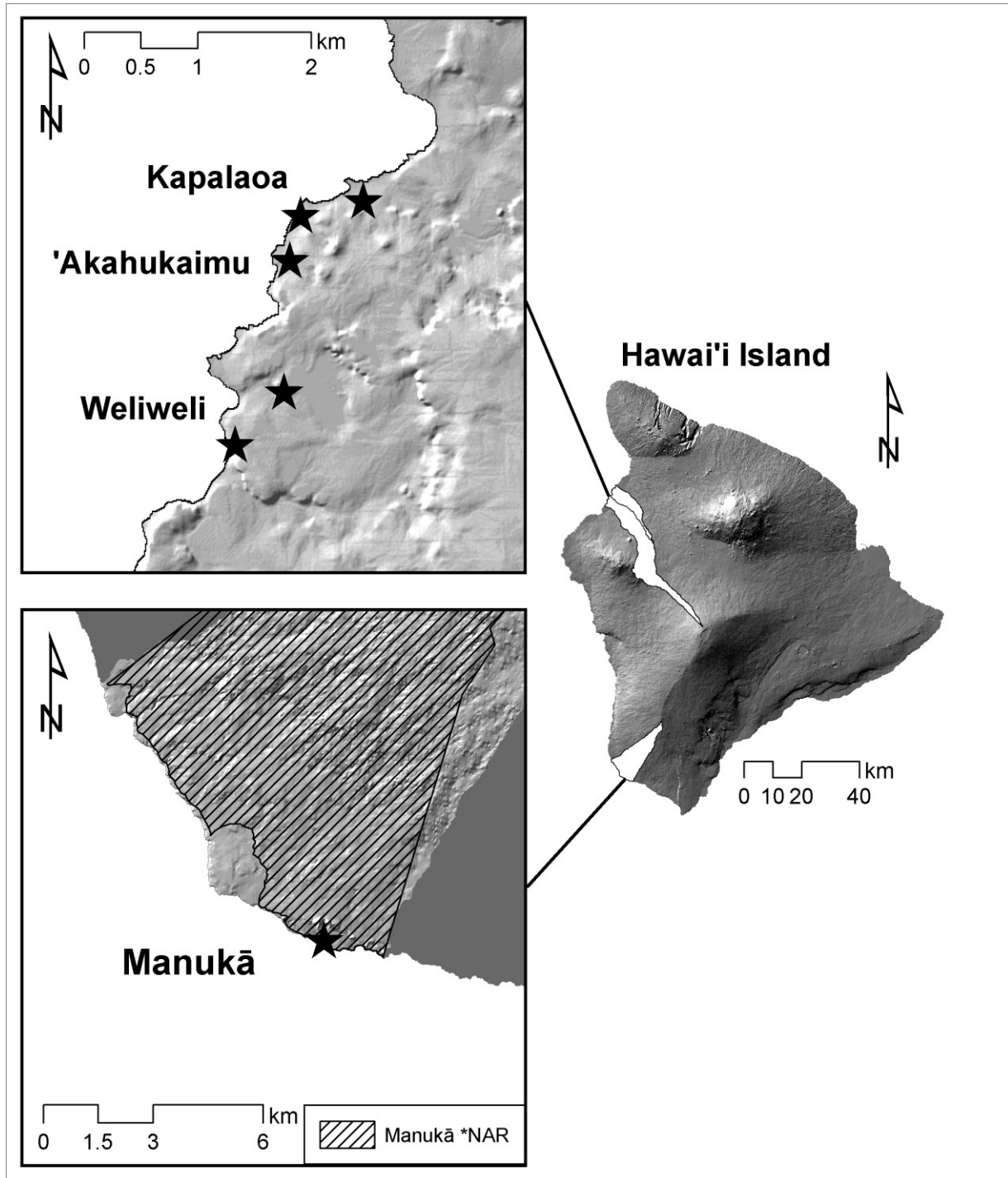


Figure 1. Locations of anchialine pool sampling sites along the western coast of Hawai'i Island.
*Natural Area Reserve.

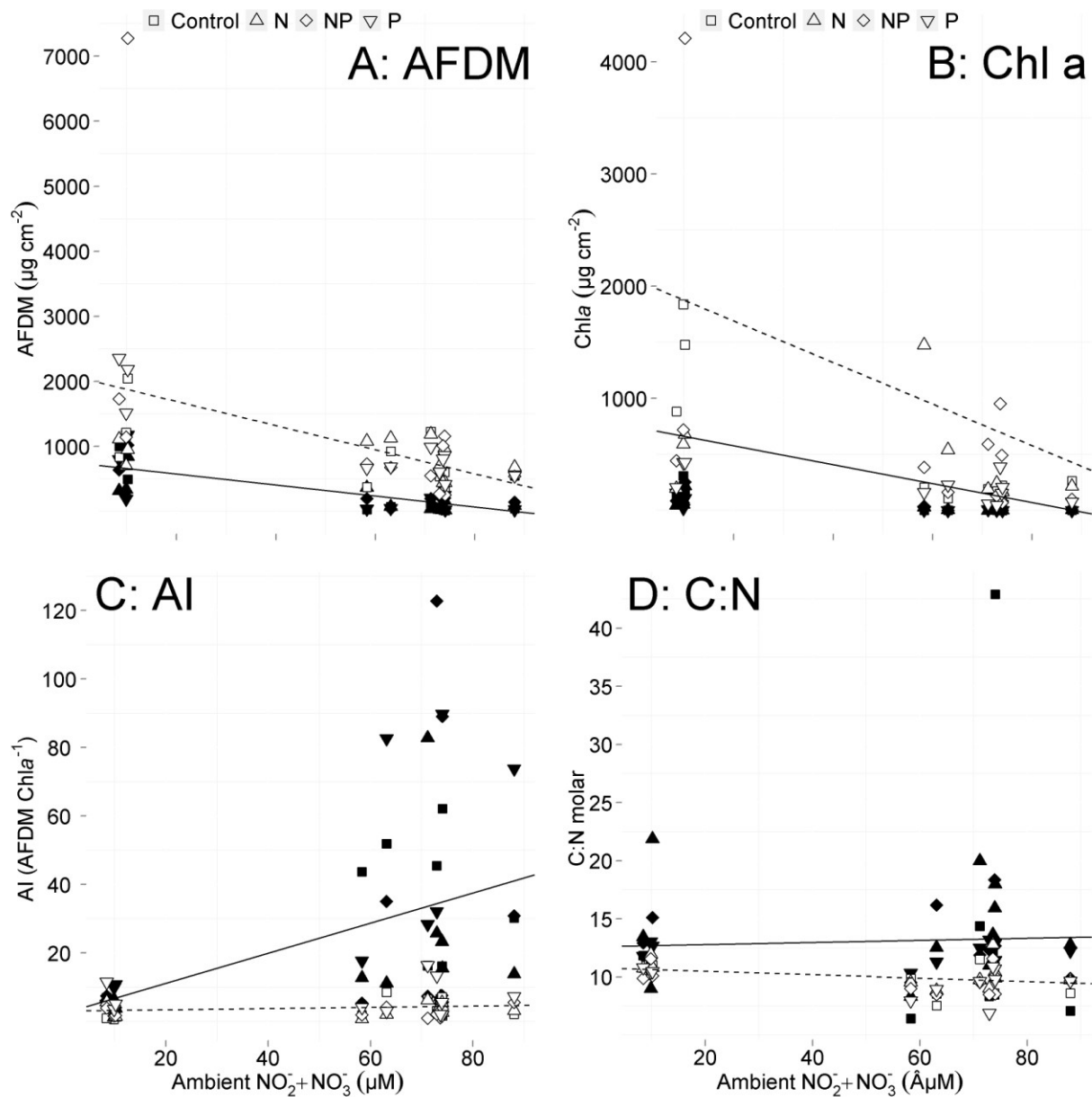


Figure 2. Microalgal response measurements for grazed (filled shapes) and ungrazed (hollow shapes) treatments across background $\text{NO}_2^- + \text{NO}_3^-$ concentrations (μM). A) ash-free dry mass (AFDM, $\mu\text{g cm}^{-2}$), B) chlorophyll *a* (Chl *a*, $\mu\text{g cm}^{-2}$), C) autotrophic index (AFDM Chl *a*⁻¹, AI), D) C:N ratio. Lines are best fit linear regressions of response variables across background $\text{NO}_2^- + \text{NO}_3^-$ concentrations for grazed (solid) and ungrazed (dashed) treatments.

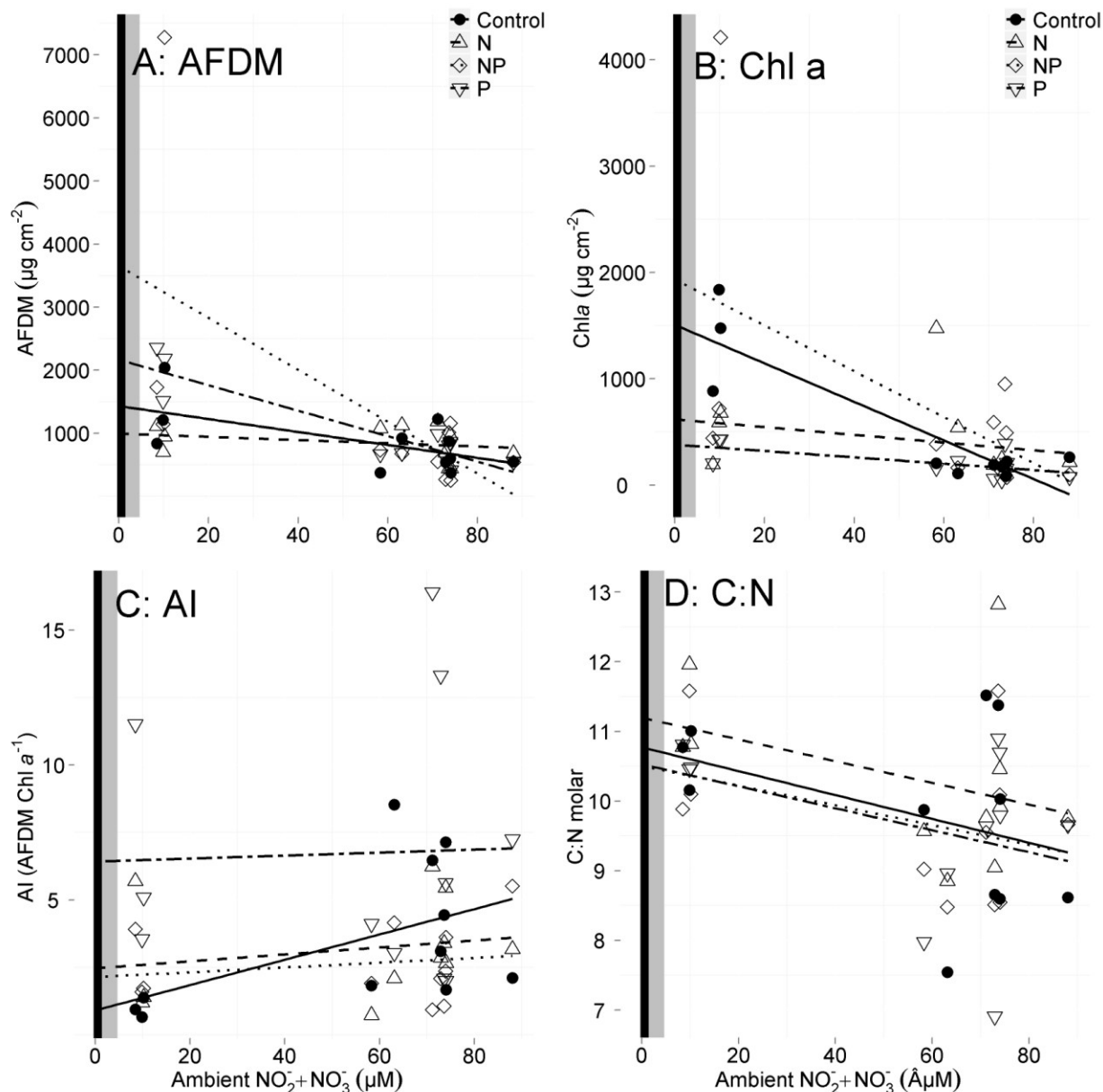


Figure 3. Microalgal response measurements for nutrient treatments across background $\text{NO}_2^- + \text{NO}_3^-$ concentrations (μM) from ungrazed epilithon samples. A) ash-free dry mass (AFDM, $\mu\text{g cm}^{-2}$), B) chlorophyll *a* (Chl *a*, $\mu\text{g cm}^{-2}$), C) autotrophic index (AFDM Chl a^{-1} , AI), D) C:N molar ratio. Lines are best fit linear regressions of response variables across background $\text{NO}_2^- + \text{NO}_3^-$ concentrations for each nutrient treatment (Control: solid, N: dash, NP: dotted, P: dot-dash). Shaded areas indicate $\text{NO}_2^- + \text{NO}_3^-$ concentrations that are within standards for Hawaiian anchialine pools used by Wiegner et al. (2006) based on measurements by Brock and Kam (1997). Solid vertical lines are background $\text{NO}_2^- + \text{NO}_3^-$ mean concentration thresholds for estuarine waters based on water quality standards by HDOH (2009).

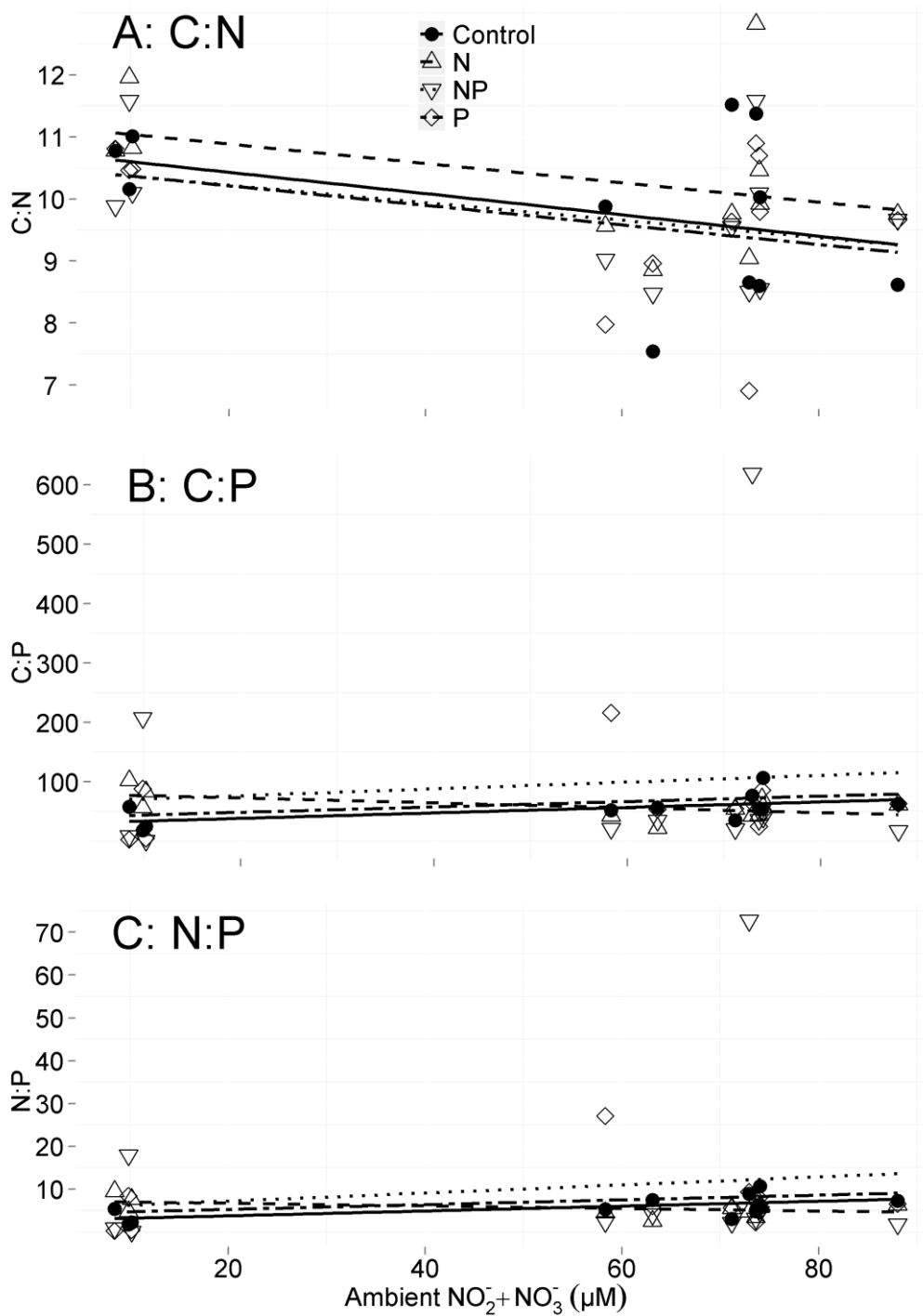


Figure 4. Stoichiometric molar ratios of carbon:nitrogen (C:N), carbon:phosphorus (C:P) and nitrogen:phosphorus (N:P) for each nutrient treatment- nitrogen (N), phosphorus (P), nitrogen+phosphorus (N+P) and control- across background $\text{NO}_2^- + \text{NO}_3^-$ concentrations (μM). A) C:N, B) C:P, and C) N:P. Lines are best fit linear regressions of response variables across background $\text{NO}_2^- + \text{NO}_3^-$ concentrations for each nutrient treatment (Control: solid, N: dash, NP: dotted, P: dot-dash).

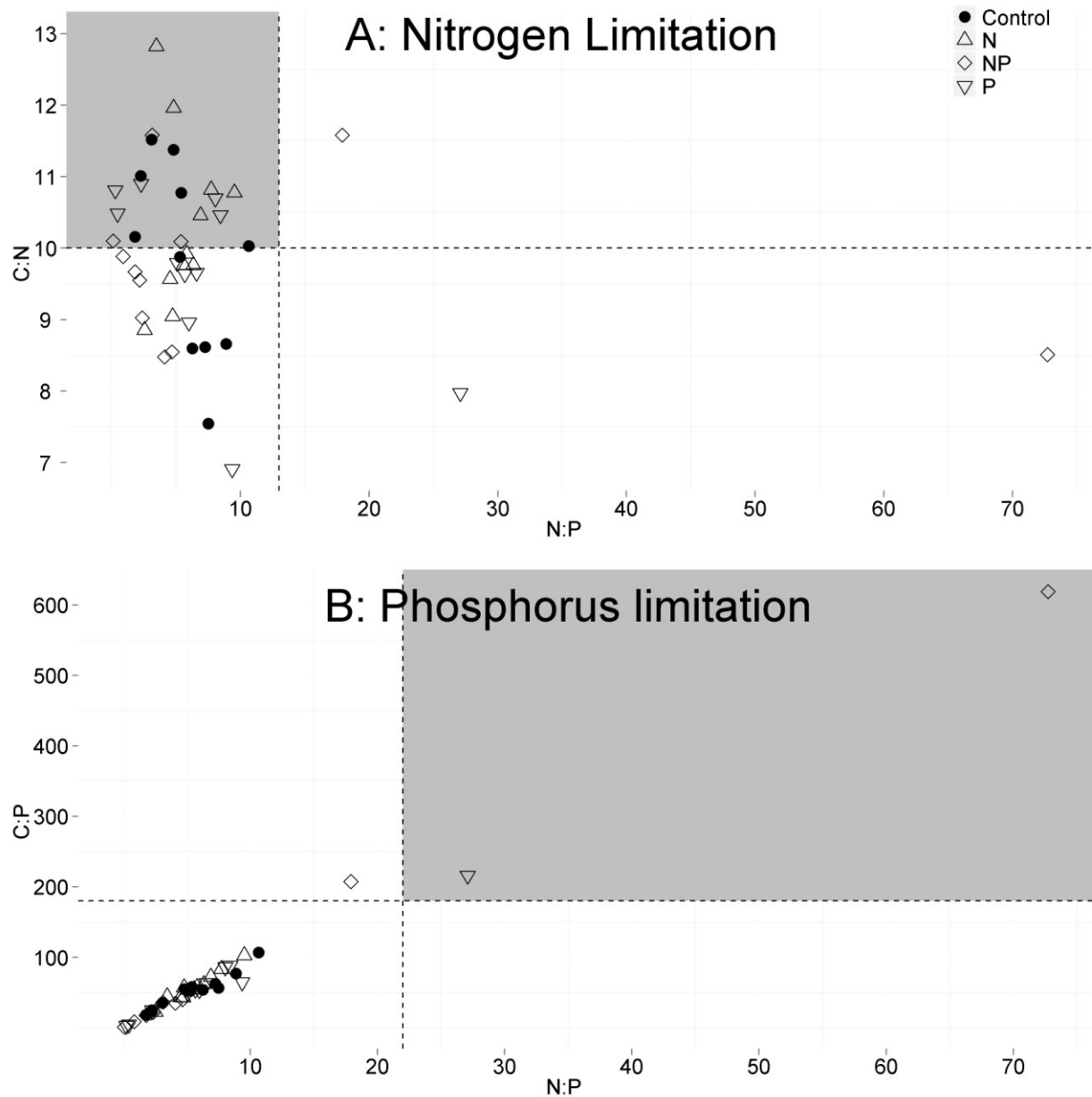


Figure 5. Nutrient stoichiometry (C:N, C:P, and N:P molar) of ungrazed epilithon samples across nutrient treatments based on nutrient limitation thresholds for benthic microalgae based on data from Hillebrand and Sommer (1999, dashed lines). Points within shaded regions of the plots suggest respective nutrient limitations for A) Nitrogen and B) Phosphorus.

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