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Responses of bloom forming and non-bloom forming macroalgae to nutrient enrichment in Hawai'i, USA

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ABSTRACT

Macroalgal blooms of *Ulva lactuca* and *Hypnea musciformis* have been problematic in shallow coastal waters around agricultural and urbanized regions of Maui, Hawai'i for decades. Observations have highlighted the correspondence between these blooms and elevated nutrient levels from the adjacent land-use, however little evidence exists regarding the effects of nutrient enrichment on the blooming and non-blooming macroalgae in the area. To determine if elevated nutrient levels influence *H. musciformis* physiology, we conducted a nutrient enrichment (+N, +P, and +N+P) experiment and measured growth, photosynthetic status, and pigment absorbance. Phycobilin pigments were significantly reduced in the no addition and +P treatment and maintained in those with N additions, suggesting that *H. musciformis* can use phycobilins to store N. We conducted a second, larger experiment with additions of secondarily-treated wastewater effluent on the bloom forming species *Acanthophora spicifera*, *H. musciformis*, and *U. lactuca* and the common non-bloom forming species, *Dictyota acutiloba*. All samples were initially depleted of potential N stores and measured for growth, photosynthetic status, and N uptake rates; *H. musciformis* and *U. lactuca* were also assessed for micro nutrient uptake, % tissue N, and $\delta^{15}N$ values. Growth rates of *D. acutiloba*, *H. musciformis*, and *U. lactuca* increased with increasing % wastewater effluent addition and concentrations of TN and NO_3^- and those of the bloom forming species were 2-fold higher. All species increased photosynthetic capacity and saturation irradiance with increasing % wastewater effluent addition and concentrations of TN and NO_3^-. *U. lactuca* was the most sensitive to low N conditions, evidenced by declines in light capturing efficiency. All species utilized a substantial amount of N over 24 h. *H. musciformis* and *U. lactuca* also (1) utilized micro nutrients: iron, manganese, molybdenum, and zinc, (2) decreased % tissue N in low N conditions, (3) increased % tissue N in response to elevated N conditions, and (4) expressed elevated $\delta^{15}N$ values with increasing additions of wastewater effluent. These results demonstrate that in Hawai'i, the bloom forming species *H. musciformis* and *U. lactuca*, have similar physiological responses to decreased and increased nutrient levels.

1. Introduction

Excess anthropogenic nutrients severely impact coastal ecosystems worldwide with two general ecosystem responses (1) declines in original habitat, including seagrass meadows in estuaries (Twilley et al., 1985; Burkholder et al., 1992; Zaitsev, 1992; Fong et al., 1993; Peckol et al., 1994) and benthic structure and composition on coral reefs (Barnes, 1973; Smith et al., 1981; Walker and Ormond, 1982; Bell, 1992; McCook, 1999; Cole et al., 2004) and (2) increases in macroalgal growth and abundance (Lapointe, 1997; Paerl, 1997; Valiela et al., 1997; Stimson et al., 2001; Morand and Merceron, 2005; Viaroli et al., 2005). Although declines in herbivore populations from over-fishing (Jackson et al., 2001; Pandolfi et al., 2003) or disease outbreaks (Hughes, 1994) would likely contribute to increases in the percent cover of macroalgae, the formation of macroalgal blooms in close proximity to urbanized and agricultural areas worldwide suggests that anthropogenic nutrient loading is the dominant factor allowing for excessive biomass production (Goreau, 1992; Peckol et al., 1994; Pedersen and Borum, 1997; Raven and Taylor, 2003).

Most macroalgal blooms consist of one or two species, suggesting that the blooming species are more responsive to excess nutrients than other macroalgae in the area. Anthropogenic nutrient driven blooms of opportunistic macroalgae in the genus *Ulva* (Chlorophyta) (referred to as "green tides") have been well documented in temperate regions of the world (Brittany, France, Briand, 1989; Puget Sound, Washington, USA, Thom and Albright, 1990; Waquoit Bay, Massachusetts, USA, Valiela et al., 1992;

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Venice, Lagoon, Italy, Sfriso et al., 1993; Ythan Estuary, Scotland, UK, Raffaelli et al., 1998; Tancada Lagoon, Ebro Delta, NE Spain, Menendez and Comin, 2000; Yellow Sea and East China, Hu et al., 2010). In temperate and tropical regions, increased eutrophication has led to biomass accumulations of opportunistic Chlorophyceaens with simple morphologies including *Ulva* (bistromatic lamina and monostromatic hollow cylinders), *Codium* (unicellular interwoven filaments), *Cladophora* (branched filament), *Chaetomorpha* (unbranched filament), and *Ulvaria* (monostromatic blades) (Fletcher, 1996; Morand and Briand, 1996; Lapointe, 1997; Valiela et al., 1997; Nelson et al., 2003; Lapointe et al., 2005a; Teichberg et al., 2010). Pedersen and Borum (1996) conclude that opportunistic macroalgae have fast growth and high nutrient uptake rates and are, therefore, able to substantially increase their biomass when exposed to excessive nutrient (nitrogen, N and phosphorus, P) levels. This implies that blooms of opportunistic macroalgae will persist in locations where nutrient levels are sustained above the threshold levels of prolific algal growth (on coral reefs for example: dissolved inorganic $N = 1.0\ \mu M$, soluble reactive $P = 0.1\ \mu M$, Bell, 1992) until such nutrients are reduced to levels that will not promote high algal growth rates.

To decrease macroalgal biomass in areas of persistent blooms it is crucial to verify the response of these species to nutrient enrichment and determine the source(s) of land-based nutrients in the area. This information can assist management officials in the decision making processes of regulating land-based nutrient loading in areas of macroalgal blooms. Globally, over the past few decades, sources of anthropogenic N have been detected by investigating the isotopic signature of N (^{15}N:^{14}N, expressed as $\delta^{15}N$; Eq. 4) (Gartner et al., 2002; Costanzo et al., 2005; Lin et al., 2007; Risk et al., 2009; Dailer et al., 2010, 2012). This is possible because different N sources have distinct $\delta^{15}N$ signatures. Naturally occurring and fertilizer N generally range from 0 to 4‰ and −4 to 4‰, respectively (Macko and Ostrom, 1994) and sewage derived wastewater N ranges from 11 to 38‰ (Kendall, 1998; Gartner et al., 2002; Savage and Elmgren, 2004) depending on the level and type of wastewater treatment. Elevated $\delta^{15}N$ values arise from the denitrification of nitrate and nitrification of ammonia, during which microbial fractionation occurs for the easier to metabolize, lighter isotope (^{14}N) (Heaton, 1986). The volatilization of ^{14}N-ammonia also enriches the sewage N source in ^{15}N relative to ^{14}N (Heaton, 1986). The release of N_2 into the atmosphere has prompted some wastewater treatment facilities (including those on the island of Maui, Hawai'i, S. Parabicoli, pers. comm.) to use a combination of denitrification and nitrification, termed Biological Nitrogen Removal, to reduce N levels of the effluent (Wiesmann, 1994). The wastewater effluent from such facilities likely has highly elevated $\delta^{15}N$ values. Macroalgae take up N from their environment with no evidence of N fractionation (Cohen and Fong, 2005), therefore their $\delta^{15}N$ values likely represent the integration of all available N sources. Macroalgae growing adjacent to sewage outfalls frequently have enriched $\delta^{15}N$ values ranging from 9 to 15‰ (Gartner et al., 2002; Costanzo et al., 2005; Lin et al., 2007). To date, the highest reported macroalgal $\delta^{15}N$ value is 50.1 ‰ from samples of *Ulva lactuca* grown over warm, freshwater seeps on a nearshore reef at Kahekili on Maui (Dailer et al., 2010). Kahekili is located near the Lahaina Wastewater Reclamation Facility (operated by the County of Maui) that utilizes Class V injection wells to dispose of 3–5 million gallons of wastewater effluent daily. The wastewater effluent from this facility has been continuously detected on this reef through high $\delta^{15}N$ values of transplanted and intertidal macroalgae (Dailer et al., 2010, 2012).

Increasing populations and substantial agricultural areas across Hawai'i subject the adjacent estuaries and coral reefs to anthropogenic nutrient loads (Laws, 2003; Stimson et al., 2001; Derse et al., 2007). In the 1970s, the increased abundance and spread of *Dictyosphaeria cavernosa* was documented in Kaneohe Bay, Oahu as a result of nutrient-rich sewage discharge to the southern region of the bay (Soegiarto, 1973; Banner, 1974; Smith et al., 1981; Pastorock and Bilyard, 1985). The abundance of *D. cavernosa* decreased after the sewage was diverted to an offshore outfall (Hunter and Evans, 1995). The role of anthropogenic N in the promotion of opportunistic green macroalgal blooms and often co-occurring red macroalgal blooms has been well documented for temperate (Björnsäter and Wheeler, 1990; Valiela et al., 1992; Fong et al., 1993; Pedersen and Borum, 1997; Fong et al., 1998; Menendez and Comin, 2000; Nelson et al., 2003; Fox et al., 2008; Teichberg et al., 2008; Thornber et al., 2008) and tropical (Smith et al., 1981; Lapointe, 1997; Lapointe et al., 2004, 2005a,b; Barile and Lapointe, 2005) regions. Studies have not specifically linked elevated N and/or P levels to blooms consisting of both *Ulva lactuca* and *Hypnea musciformis* (Rhodophyta) in Hawai'i.

Blooms primarily comprised of *H. musciformis* and *U. lactuca* are problematic in shallow, coastal waters around urbanized and agricultural regions of Maui and annually cost over 20 million dollars in economic losses (Van Beukering and Cesar, 2004). Another species commonly found in these blooms is *Acanthophora spicifera*, which is the most widespread and successful alien invasive alga in Hawai'i (Smith et al., 2002). These macroalgal blooms occur in the following four regions of Maui across the corresponding length of coastline: (1) northwest, ~7.0 km, (2) central-south, ~2.4 km, (3) southwest, ~10.5 km, and (4) central-north, ~11.3 km (blooms in this region occur during summer months and are decimated with large winter swells) (Fig. 1; West Maui Watershed Owners Manual, 1997; MD per. obs.). The co-occurrence of green and red macroalgal blooms suggests that bloom forming species from different algal phyla may respond similarly to increased N and/or P levels even though their pigment composition differs. Typically in low light conditions, red macroalgae assemble dark colored phycobilin pigments phycoerythrin (PE) and phycocyanin (PC), which require more N to construct and absorb more light than chlorophyll complexes (Graham and Wilcox, 2000). However, as documented for *Gracilaria* spp., PC and PE can also be used to store N (Ryther et al., 1981; Lapointe and Duke, 1984; Horrocks et al., 1995). On Maui, blooms of *H. musciformis* consist of plants that are dark purple in high light conditions, suggesting that phycobilin pigments are likely used for N storage.

To investigate the role of N and P on the growth, photosynthetic properties, and pigment composition of *H. musciformis* we performed a preliminary nutrient enrichment experiment with the following treatments: no addition, $+NH_4^+$, $+PO_4^{3-}$, and $+PO_4^{3-}$ and $+NH_4^+$. Based on the results from the preliminary experiment and the detection of wastewater effluent in areas of *H. musciformis*

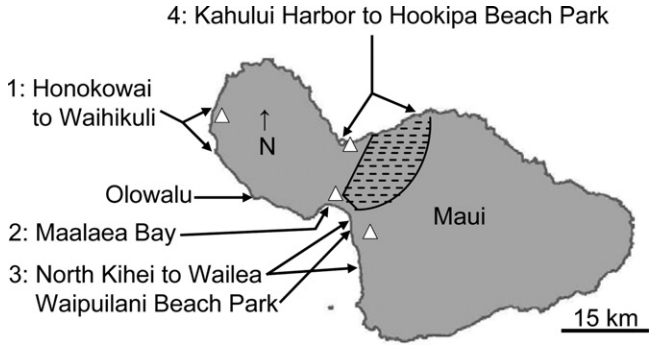

Fig. 1. Long-term (decadal) locations of *Hypnea musciformis* and *Ulva lactuca* blooms on Maui, Hawai'i. Region 1 has fluctuations in macroalgal biomass, regions 2 and 3 have persistent blooms, and region 4 blooms are subject to large winter swells and primarily occur in the summer months. White triangles represent wastewater injection well locations and the dashed area represents ongoing agricultural (sugar cane) operations.

and *Ulva lactuca* blooms on Maui (Dailer et al., 2010), we adjusted our methods and conducted a larger experiment with incremental additions of secondarily-treated wastewater effluent, containing an assortment of micro nutrients and elevated N levels relative to oligotrophic coral reef conditions. These experiments were conducted on the following four species: *Dictyota acutiloba* (non-bloom forming), and the bloom forming species, *Acanthophora spicifera*, *H. musciformis*, and *U. lactuca*. To determine if these species would physiologically respond similarly to increasing nutrient levels, we exposed samples to a gradient of wastewater effluent and measured their growth rates, photosynthetic properties, and N uptake rates. For *H. musciformis* and *U. lactuca*, additional analyses were performed to determine if they (1) also use micro nutrients, (2) decrease % tissue N in low N conditions, (3) increase % tissue N in high N conditions, and (4) would reflect the associated isotopic signatures ($\delta^{15}N$) from the wastewater N source.

2. Materials and methods

2.1. Preliminary experiment: response of Hypnea musciformis to N and P enrichment

Samples of *Hypnea musciformis* were collected in July 2006 from Kaimana Beach Park in Honolulu, Oahu (an urbanized area with blooms of *H. musciformis* and *Ulva lactuca*) and transported to the University of Hawai'i, Mānoa. Samples (n = 3 per treatment) were blotted with a towel to remove excess water, trimmed to weigh ~0.400 g, and assessed for initial photosynthetic status with Pulse Amplitude Modulated fluorometry (PAM; see Section 2.3) and pigment composition with *in vivo* absorbance spectra (Shimadzu UV Vis-2101 spectrophotometer with a Shimadzu 150 mm integrating sphere attachment for macroalgal tissues 0.5 cm × 1.0 cm in dimension; Beach et al., 2000). Samples were transported to the Anuenue aquaculture facility (in Honolulu), and randomly placed in 1.0 L glass beakers (n = 1 per beaker) with individual aerators. Beakers were housed in a shaded (maximum light levels of 500 μmol m^{-2} s^{-1} PAR, measured with a 4π quantum LiCor light sensor) open air tank with a running seawater bath to prevent the treatment waters from heating. Samples were subjected to one of four treatments for seven days: no addition, +P (~4.5 μmol PO_4^{3-}), +N (~300 μmol NH_4^+) and +N+P (~300 μmol NH_4^+ and ~4.5 μmol PO_4^{3-}). N concentrations were chosen based on levels reported for blooms of *H. musciformis* and *U. lactuca* on Maui (277 DIN; Hunt and Rosa, 2009). P concentrations were based on previous saturation experiments (6–12 μ mol PO_4^{3-}; Björnsäter and Wheeler, 1990). Treatment waters in each 1.0 L beaker were changed daily to maintain nutrient concentrations. Water samples were collected (n = 3 per treatment) to verify nutrient additions on days 0 and 7. On the seventh day, samples were assessed for final photosynthetic status with PAM fluorometry (see Section 2.3), weighed, transported to the University of Hawai'i, Mānoa, and analyzed for final pigment composition with *in vivo* absorbance. Growth data are expressed as specific growth rates (% d^{-1}), calculated as follows, with biomass (N) in g and time (t) in days (Lobban and Harrison, 1994):

$$\mu = \frac{100[\ln(N_{final}/N_{initial})]}{t} \quad (1)$$

2.2. Main experiment: responses of Acanthophora spicifera, Dictyota acutiloba, Hypnea musciformis, and Ulva lactuca to a gradient of wastewater effluent

This study was conducted on the following four species during the corresponding month in 2008 at the University of Hawai'i, Lahaina field station on Maui: *Acanthophora spicifera* (September), *Dictyota acutiloba* (October), *Hypnea musciformis* (June), and *Ulva lactuca* (July). Prior to experimental trials, all species were acclimated to low N seawater from Olowalu (a rural area that currently has no anthropogenic nutrient sources) (Fig. 1) to deplete potential internal N stores. Algal samples were collected from Waipuilani Beach Park, transported to the Lahaina field station and randomly placed in 1.0 L beakers with individual aerators (n = 1 per beaker) in a shaded (maximum light levels *ca* 500 μ mol m^{-2} s^{-1} PAR, measured with a 4π quantum LiCor light sensor) outdoor aquarium system in water baths to prevent the seawater from heating. Low N acclimation occurred for seven days (Fong et al., 1994), during which the seawater in each beaker was changed every two days.

Low N acclimated samples were assessed for initial photosynthetic status with Pulse Amplitude Modulated fluorometry (PAM; see Section 2.3), blotted with a towel to remove excess water, trimmed to weigh ~0.400 g, photographed, and added to one of the following seven treatments (n = 6 per treatment): (1) no addition and wastewater effluent additions of (2) 25 ml (2.5%), (3) 50 ml (5.0%), (4) 75 ml (7.5%), (5) 100 ml (10.0%), (6) 150 ml (15.0%), and (7) 200 ml (20.0%) to create a final volume of 1.0 L. Each sample was housed in a 1.0 L beaker provided with an aerator in the abovementioned Lahaina field station aquarium system. The wastewater effluent was a clear liquid obtained at the beginning of each trial from the Lahaina Wastewater Reclamation Facility where it was secondarily treated and disinfected with chlorine. All treatment waters were changed daily with (1) low N water from Olowalu, (2) the corresponding addition of wastewater effluent, and (3) the appropriate addition of natural sea salt to maintain a constant and representative salinity (35‰; confirmed with a 7-Multi conductivity meter model 8603, Mettler-Toledo, Switzerland, calibrated with Mettler-Toledo conductivity standards). Based on the observations of Naldi and Wheeler (2002) that *U. fenestra* and *Gracilaria pacifica* had the highest growth response to increased nutrients over the first nine days and the preliminary observations of *H. musciformis* in low N conditions for seven days; we elected to run trials for nine days in an attempt to observe a physiologically complete response of the algae to the nutrient gradient. On the ninth day, samples were assessed for final photosynthetic status with PAM fluorometry (see Section 2.3) weighed, and photographed. All growth data are expressed as specific growth rates (% d^{-1}) (Eq. (1)).

2.3. Photosynthetic measurements

Samples were assessed for photosynthetic status with Pulse Amplitude Modulated (PAM) fluorometry (Diving PAM, Walz), which measures *in vivo* chlorophyll fluorescence. Measurements occurred between 1100 h and 1500 h to minimize variation associated with diurnal changes of photosynthetic activity. Rapid Light Curves (RLCs) were used to determine the following photosynthetic parameters (1) relative maximum Electron Transport Rate ($rETR_{max}$; maximum photosynthetic capacity), (2) α, the slope of the light limited region of the curve (proportional to the efficiency of light capture), and (3) minimum photosynthetic saturation irradiance (E_k; optimal irradiance for maximal electron transport), which is determined by finding the interception of α with the maximum photosynthetic rate ($E_k = rETR_{max}/\alpha$) (Ralph and Gademann, 2005). PAM was used to assess samples while connected to a laptop to provide instantaneous observations of the RLCs. The actinic light factor was adjusted to the photosynthetic capacity of the sample to obtain RLCs prior to photoinhibition. All photosynthetic parameters were calculated with the methods provided by Platt et al. (1980), which resulted in reliable parameter estimates (<0.5% variation attributable to error).

2.4. Nutrient concentrations and uptake rates

Nutrient uptake rates were determined over a 24 h period from day eight to nine to obtain rates that would be applicable to ongoing blooms of *Hypnea musciformis* and *Ulva lactuca*. For all species, uptake rates were determined for total organic carbon (TOC), total nitrogen (TN), nitrate (NO_3^-), and nitrite (NO_2^-). For *H. musciformis* and *U. lactuca* additional uptake rates were determined for total phosphorus (TP), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), and zinc (Zn). On days eight and nine, algal samples ($n = 3$ per treatment) were weighed and corresponding water samples were collected with sterile syringes (prior to the return of the algal sample on day eight), filtered through sterile 0.45 μm nylon filters into acid washed bottles and frozen. Frozen water samples were sent to the Analytical Laboratory, University of Hawai'i, Hilo and analyzed with the following instrumentation for the corresponding nutrients: Nutrient AutoAnalyzer, NO_3^- and NO_2^- (Marti and Hale, 1981); Shimadzu TOC/TN, TOC and TN; Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES), TP, Cu, Fe, Mn, Mo, and Zn (Garbarino et al., 1989). TP was measured instead of soluble reactive P because it is applicable to the State of Hawai'i water quality standards. All quality assurance indicators were acceptable (standard curves, lab spikes, certified reference materials, and digestion spikes). Nutrient uptake rates ($g^{-1}\ d^{-1}$) and % change in the nutrient concentrations were calculated as:

$$\text{uptake of nutrient } X = \frac{(X_{D8} - X_{D9})/N_{D9}}{t} \quad (2)$$

$$\%\ \text{change nutrient } X = \frac{X_{D8} - X_{D9}}{X_{D8}} \times 100 \quad (3)$$

where X_{D8} = concentration of nutrient X on day eight; X_{D9} = concentration of nutrient X on day nine; N_{D9} = biomass of the algal sample on day nine; $t = 1$ day.

2.5. % tissue N and $\delta^{15}N$ values of *Hypnea musciformis* and *Ulva lactuca*

Samples of *Hypnea musciformis* and *Ulva lactuca* ($n = 3$ per species per condition/treatment) were prepared for the analysis of % tissue N and $\delta^{15}N$ on the day of field collection (initial bloom levels), the first day of the experimental trials (low N acclimated) and on the ninth day of exposure to wastewater effluent additions. Samples were dried at 60 °C to a constant weight, ground with mortar and pestle into a powder, and sent for mass spectrometer analysis of % tissue N and $\delta^{15}N$ to the Biogeochemical Stable Isotope Laboratory, University of Hawai'i, Mānoa. Samples were weighed then analyzed with a Carlo Erba NC 2500 Elemental Analyzer, Finnigan MAT ConFloII, and Finnigan MAT DeltaS (with source upgrade). Ratios of ^{15}N:^{14}N were expressed relative to atmospheric N and calculated as (Peterson and Fry, 1987):

$$\delta^{15}N(‰) = \left\{\frac{R_{sample}}{R_{standard}} - 1\right\} \times 10^3, \text{ where } R = \frac{^{15}N}{^{14}N} \quad (4)$$

2.6. Statistical analyses

Preliminary experiment: *T*-tests were used to determine if there were significant differences in nutrient concentrations between the no addition and enrichment treatments. All data were normally distributed and displayed homogeneity of variances. Growth rates ($\%\ d^{-1}$) were analyzed with a one-way ANOVA. A two-way ANOVA with treatment and day as predictive factors was performed on the initial and final values of $rETR_{max}$, α, E_k, PC, and PE. If a significant result was obtained for the two-way ANOVA, a Tukey's *post hoc* multiple comparisons test was performed to assess differences in final values for each variable between treatments.

Main experiment: All data were normally distributed and displayed homogeneity of variances. Data were examined with regressions (simple, power, and hyperbolic where appropriate) with % wastewater effluent addition and concentrations of total nitrogen (TN) and nitrate (NO_3^-) as predictor variables. TN and NO_3^- concentrations were selected from the other nutrients because they increased the most with increasing wastewater effluent addition for all trials and provide information that is applicable to management officials. The following dependent variables were regressed with % wastewater effluent addition and concentrations of TN and NO_3^-: (1) growth rate ($\%\ d^{-1}$), $rETR_{max}$, E_k, and α, for all species, and (2) % tissue N and $\delta^{15}N$ values of *Hypnea musciformis* and *Ulva lactuca*. *T*-tests were used to determine significant differences in: (1) initial and final α values of *U. lactuca* in the no addition, (2) day nine wet weights between the no addition and wastewater effluent additions, (3) day eight water chemistry and nutrient uptake rates between the no addition and additions of wastewater effluent, and (4) % tissue N and $\delta^{15}N$ values between field and low N acclimated samples of *H. musciformis* and *U. lactuca*. Statistics were performed with Statistica 6.0 and SigmaPlot 9.0.

3. Results

3.1. Preliminary experiment: response of *Hypnea musciformis* to N and P enrichment

The nutrient additions significantly increased the concentration of the nutrient provided compared to the no addition (NA) (Table 1). Significant effects (ANOVA, $F = 8.46$, $P = 0.007$) were found between growth rate and nutrient treatment. Growth rates ($\%\ d^{-1}$) in the +P treatment were significantly lower than those in the +N and +N+P treatments. NA growth rates were statistically similar to all nutrient treatments (Table 1). A significant interaction (two-way ANOVA, $F = 6.52$, $P < 0.00001$) was found with day by treatment. Significant increases in E_k occurred in all treatments (Table 1), the highest E_k values (193 ± 2.8) were

Table 1
Hypnea musciformis nutrient enrichment concentrations (average of initial and final per treatment), growth rate (GR, $\%\ d^{-1}$), and initial and final values of: photosynthetic capacity ($rETR_{max}$), light capturing efficiency (α), photosynthetic saturation irradiance (E_k), phycoerythrin (PE, 563:680 nm), and phycocyanin (PC, 625:680 nm) (means ± SE). Significant differences between (1) the no addition and nutrient concentration and (2) initial and final values are in bold. Significant ($P < 0.01$) differences in final values between treatments are represented by different letters.

	No Addition	+P	+N	+N+P
NH_4^+ (μM)	0.48 ± 0.22	0.53 ± 0.12	$325 \pm 34.2^{**}$	$300 \pm 30.5^{**}$
PO_4^{-3} (μM)	0.07 ± 0.02	$4.6 \pm 0.2^{***}$	0.12 ± 0.03	$4.6 \pm 0.1^{***}$
GR ($\%\ d^{-1}$)	$9.51 \pm 0.4^{a,b}$	8.28 ± 0.2^{b}	11.0 ± 0.2^{a}	11.2 ± 0.3^{a}
$rETR_{max}$				
Initial	27.5 ± 2.0	28.5 ± 4.3	30.6 ± 2.6	26.7 ± 0.5
Final	45.0 ± 0.7	46.1 ± 1.9	39.7 ± 0.6	$52.6 \pm 0.8^{**}$
α				
Initial	0.40 ± 0.02	0.38 ± 0.03	0.40 ± 0.01	0.44 ± 0.02
Final	0.34 ± 0.01	0.33 ± 0.01	0.30 ± 0.02	$0.27 \pm 0.01^{*}$
E_k				
Initial	68.3 ± 3.6	73.4 ± 7.4	77.2 ± 7.7	62.0 ± 2.2
Final	$132 \pm 5.6^{**a}$	$139 \pm 4.0^{**a}$	$133 \pm 6.3^{*a}$	$193 \pm 2.8^{***b}$
PE				
Initial	0.98 ± 0.01	0.97 ± 0.003	0.96 ± 0.003	0.98 ± 0.001
Final	$0.69 \pm 0.03^{***a}$	$0.77 \pm 0.02^{***a}$	0.99 ± 0.001^{b}	1.0 ± 0.002^{b}
PC				
Initial	0.78 ± 0.01	0.77 ± 0.01	0.77 ± 0.01	0.80 ± 0.01
Final	$0.58 \pm 0.01^{***a}$	$0.63 \pm 0.01^{***a}$	0.79 ± 0.004^{b}	0.82 ± 0.004^{b}

* $P < 0.05$.
** $P < 0.005$.
*** $P < 0.0005$.

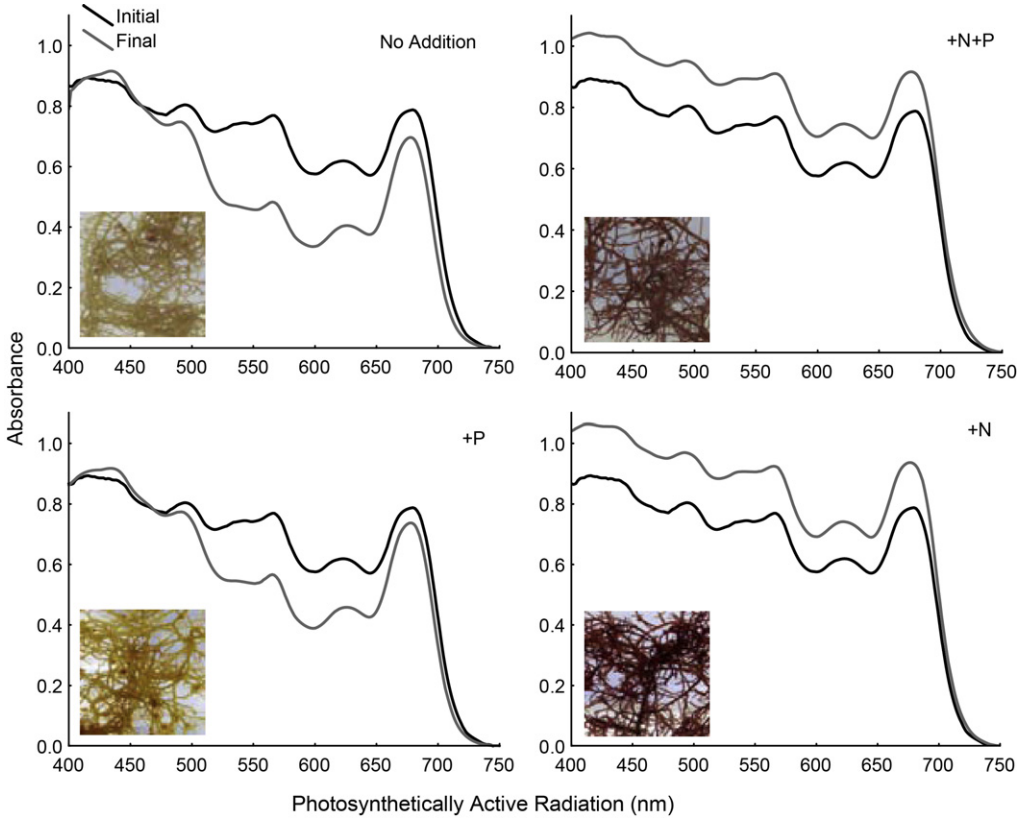

Fig. 2. Initial (black lines) and final (gray lines) *in vivo* absorbance spectra and final photographs of *Hypnea musciformis* in the no addition and enrichment treatments of +P, +N, and +N+P.

observed in the +N+P treatment and were significantly higher than all other treatments (Table 1). Samples in the +N+P treatment also had the only significant increase in $rETR_{max}$ (Table 1). Final *in vivo* absorbance spectra showed that samples in the NA and +P treatments had decreased absorbance levels in the spectra range for phycoerythrin (PE, 563 nm) and phycocyanin (PC, 625 nm) (Fig. 2). Final values of PE and PC absorbance normalized to chlorophyll *a* (680 nm) were significantly decreased from initial values in the NA and +P treatments (Table 1). These values were also significantly lower than those of samples provided with N additions, where no change in PE or PC absorbance occurred (Fig. 2, Table 1).

3.2. Main experiment: response of *Acanthophora spicifera, Dictyota acutiloba, Hypnea musciformis, and Ulva lactuca* to a gradient of wastewater effluent

3.2.1. Visual and growth response

In nine days, samples of *Acanthophora spicifera*, *Dictyota acutiloba*, *Hypnea musciformis*, and *Ulva lactuca* visibly responded to the gradient of wastewater effluent (and associated increasing concentrations of TN and NO_3^-) with darkened coloration, while no change was observed in the no addition (Fig. 3). No significant relationship was found between the growth rates (% d^{-1}) of *A. spicifera* and increasing % wastewater effluent addition (Fig. 4) or TN concentration (Table 2); however a weak but significant relationship was found with increasing NO_3^- concentration (Table 2). Significant relationships were found between the growth rates of *D. acutiloba*, *H. musciformis*, and *U. lactuca* and increasing % wastewater effluent addition (Fig. 4) and concentrations of TN and NO_3^- (Table 2). The highest growth rates observed were those of *H. musciformis* ($15.8 \pm 0.37\%$ d^{-1}) and *U. lactuca* ($15.5 \pm 0.34\%$ d^{-1}) in wastewater effluent additions of 20.0% and 10.0%, respectively. These growth rates were about 2-fold higher than the highest rates observed of *A. spicifera* ($6.60 \pm 0.40\%$ d^{-1}) and *D. acutiloba* ($7.41 \pm 0.11\%$ d^{-1}), which occurred in wastewater effluent additions of 7.5% and 20.0%, respectively. The lowest growth rates of *H. musciformis* ($3.89 \pm 0.16\%$ d^{-1}) and *U. lactuca* ($4.28 \pm 0.20\%$ d^{-1}) occurred in the no addition. The growth rate of *H. musciformis* depleted of N stores then placed in the no addition was nearly 3-fold lower than that of the no addition in the preliminary experiment ($9.50 \pm 0.41\%$ d^{-1}) and 5-fold lower than treatments with ≥10.0% wastewater effluent (~15% d^{-1}).

3.2.2. Photosynthetic response

The final $rETR_{max}$ values of all species significantly increased with increasing % wastewater effluent addition (Fig. 5a–d) and concentrations of TN and NO_3^- (Table 2). The highest observed $rETR_{max}$ values were those of *Acanthophora spicifera* (67.8 ± 4.67) and *Ulva lactuca* (90.8 ± 3.32) in wastewater effluent additions of 15.0% and 20.0%, respectively. No significant relationships were found between the final α values of *A. spicifera*, *Dictyota acutiloba*, and *Hypnea musciformis* and increasing % wastewater effluent addition (Fig. 5e–g) and concentrations of TN and NO_3^- (Table 2). Significant relationships were found between the final α values of *U. lactuca* with increasing % wastewater effluent addition (Fig. 5h) and concentrations of TN and NO_3^- (Table 2). These significant relationships were primarily due to a significant decline in α (initial: 0.274 ± 0.006, final: 0.172 ± 0.013, $P < 0.01$) in the no addition. Significant relationships were found between the final minimum photosynthetic saturation irradiance (E_k) values of *A. spicifera*, *D. acutiloba*, and *H. musciformis* and increasing % wastewater effluent addition (Fig. 5i–k) and concentrations of TN and NO_3^- (Table 2). The highest final E_k values observed were those of *U. lactuca* in the highest addition of wastewater effluent (346 ± 22.1) and in the no addition (322 ± 19.8) (Fig. 5l). However, the high final E_k values of *U. lactuca* in the no addition were the product of the decline in α rather than an

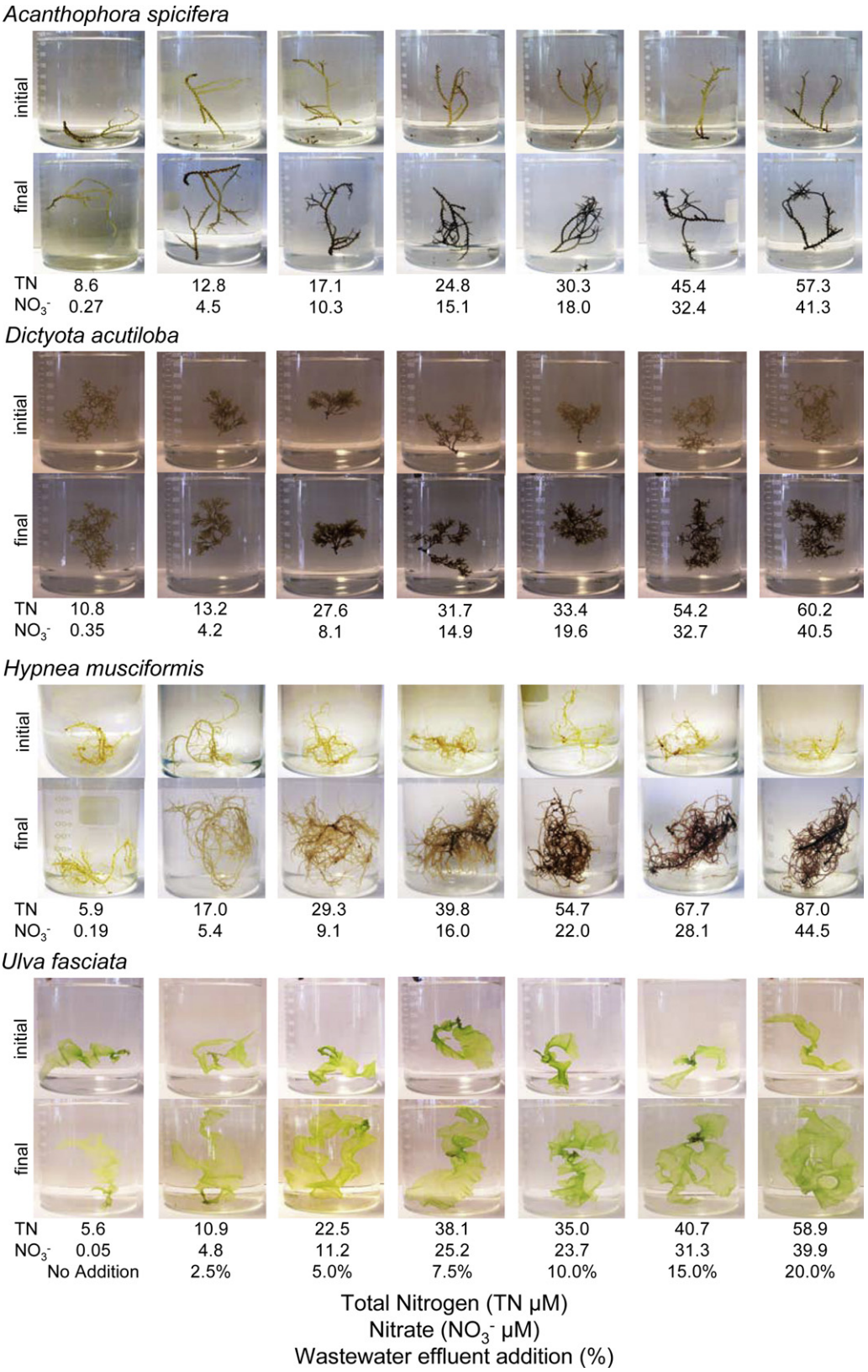

Fig. 3. Initial and final photographs of *Acanthophora spicifera*, *Dictyota acutiloba*, *Hypnea musciformis*, and *Ulva lactuca* with day 8 total nitrogen (TN) and nitrate (NO_3^-) concentrations (µM) for each addition of wastewater effluent (%).

increase in minimum photosynthetic saturation irradiance from initial values. E_k is calculated by dividing $rETR_{max}$ by α and samples in the no addition on day 9 had unchanged $rETR_{max}$ values (initial: 53.2 ± 3.09, final: 54.7 ± 2.89); therefore the decline in α consequently produced misleading, higher E_k values. However, this was the only situation where the calculation of E_k was misleading; therefore, we support the view that using RLCs to assess the photosynthetic status of samples is a successful, robust method.

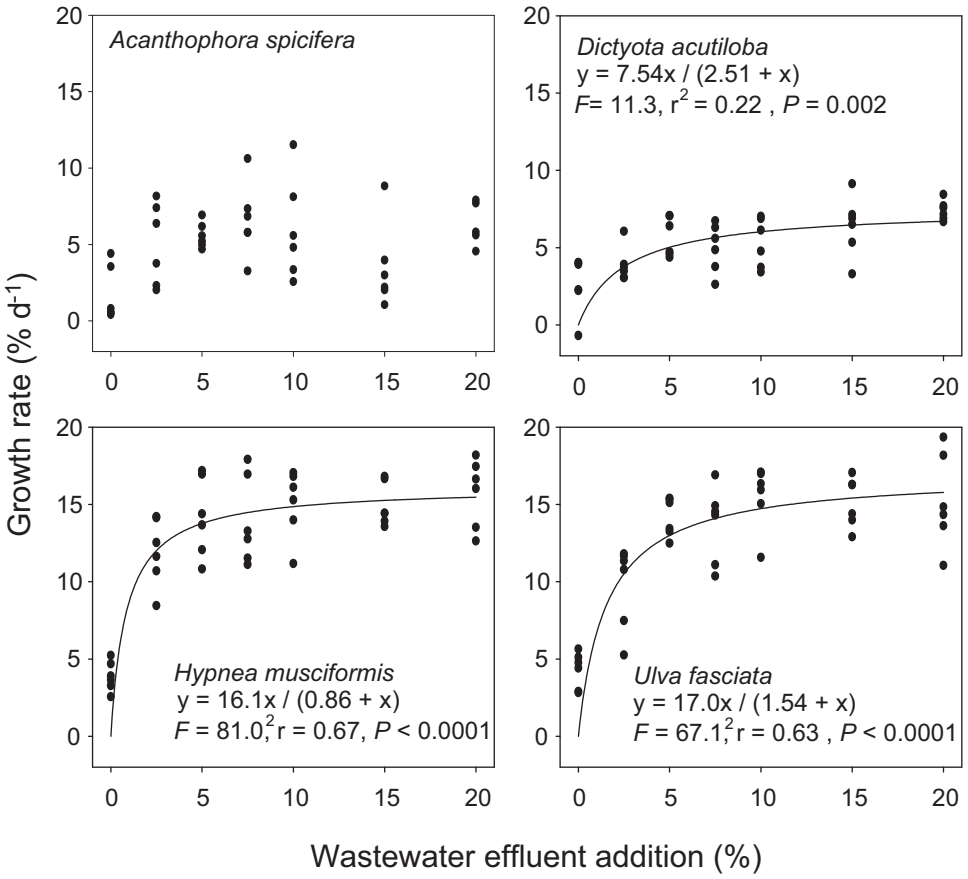

Fig. 4. Growth rate (% d^{-1}) responses of *Acanthophora spicifera*, *Dictyota acutiloba*, *Hypnea musciformis*, and *Ulva lactuca* to increasing wastewater effluent addition (%).

Table 2
Results for *Acanthophora spicifera*, *Dictyota acutiloba*, *Hypnea musciformis*, and *Ulva lactuca* from simple, power, and hyperbolic regression analyses with total nitrogen (TN) and nitrate (NO_3^-) concentration as the predictor variable for growth rate (GR, % d^{-1}), photosynthetic capacity ($rETR_{max}$), light capturing efficiency (α), and photosynthetic saturation irradiance (E_k). *H. musciformis* and *U. lactuca* relationships for % tissue N (% N) and $\delta^{15}N$ values are also presented. Dashed lines represent no significant relationship.

		Acanthophora spicifera				*Dictyota acutiloba*			
		Eq.	*F*	r^2	*P*	Eq.	*F*	r^2	*P*
TN	GR (% d^{-1})	–	–	–	–	$y = 9.79x/(24.8+x)$	38.5	0.49	<0.0001
	$rETR_{max}$	$y = 14.7x^{0.39}$	25.5	0.44	<0.0001	$y = 30.9 + 27x$	8.51	0.21	0.006
	α	–	–	–	–	–	–	–	–
	E_k	$y = 64.1x^{0.33}$	27.3	0.45	<0.0001	$y = 58.3x^{0.21}$	7.55	0.19	0.010
NO_3^-	GR (% d^{-1})	$y = 5.41x/(0.48+x)$	7.95	0.17	0.007	$y = 3.09x^{0.22}$	36.0	0.47	<0.0001
	$rETR_{max}$	$y = 30.4x^{0.22}$	34.1	0.51	<0.0001	$y = 34.1 + 0.33x$	7.76	0.19	0.009
	α	–	–	–	–	–	–	–	–
	E_k	$y = 121.9x^{0.18}$	35.7	0.52	<0.0001	$y = 101.2 + 1.03x$	6.84	0.17	0.013
		Hypnea musciformis				*Ulva fasciata*			
		Eq.	*F*	r^2	*P*	Eq.	*F*	r^2	*P*
TN	GR (% d^{-1})	$y = 18.5x/(12.6+x)$	108.7	0.73	<0.0001	$y = 19.5x/(12.2+x)$	103.6	0.72	<0.0001
	$rETR_{max}$	$y = 28.7 + 0.30x$	29.9	0.48	<0.0001	$y = 51.4 + 0.66x$	34.8	0.51	<0.0001
	α	–	–	–	–	$y = 0.15x^{0.18}$	20.4	0.38	<0.0001
	E_k	$y = 124.1 + 1.48x$	39.7	0.55	<0.0001	–	–	–	–
	%N	$y = 3.14x/(17.1+x)$	25.3	0.57	<0.0001	$y = 0.516 + 0.03x$	135.2	0.88	<0.0001
	$\delta^{15}N$	$y = 31.x/(14.8+x)$	505.5	0.96	<0.0001	$y = 41.5x/(19.8+x)$	201.7	0.91	<0.0001
NO_3^-	GR (% d^{-1})	$y = 15.2x/(0.74+x)$	128.4	0.76	<0.0001	$y = 7.93x^{0.19}$	116.3	0.74	<0.0001
	$rETR_{max}$	$y = 31.1 + 0.60x$	34.6	0.51	<0.0001	$y = 55.5 + 0.81x$	30.0	0.48	<0.0001
	α	–	–	–	–	$y = 0.22x^{0.09}$	40.6	0.55	<0.0001
	E_k	$y = 137.1 + 2.82x$	41.7	0.56	<0.0001	–	–	–	–
	%N	$y = 1.24x^{0.20}$	20.9	0.52	0.0002	$y = 0.66 + 0.04x$	215.8	0.92	<0.0001
	$\delta^{15}N$	$y = 12.4x^{0.21}$	306.6	0.94	<0.0001	$y = 11.7x^{0.26}$	536.7	0.97	<0.0001

3.2.3. Nutrient concentrations, final wet weights, and uptake rates

In all trials, significant increases in N concentrations of day 8 water samples occurred with increasing additions of wastewater effluent for total nitrogen (TN), nitrate (NO_3^-), and nitrite (NO_2^-; with the exception of the *Acanthophora spicifera* trial, where NO_2^- was not detected) (Tables 3–5). The day 8 water chemistry for the *Hypnea musciformis* and *Ulva lactuca* trials also had significant increases in concentrations of total phosphorous (TP)

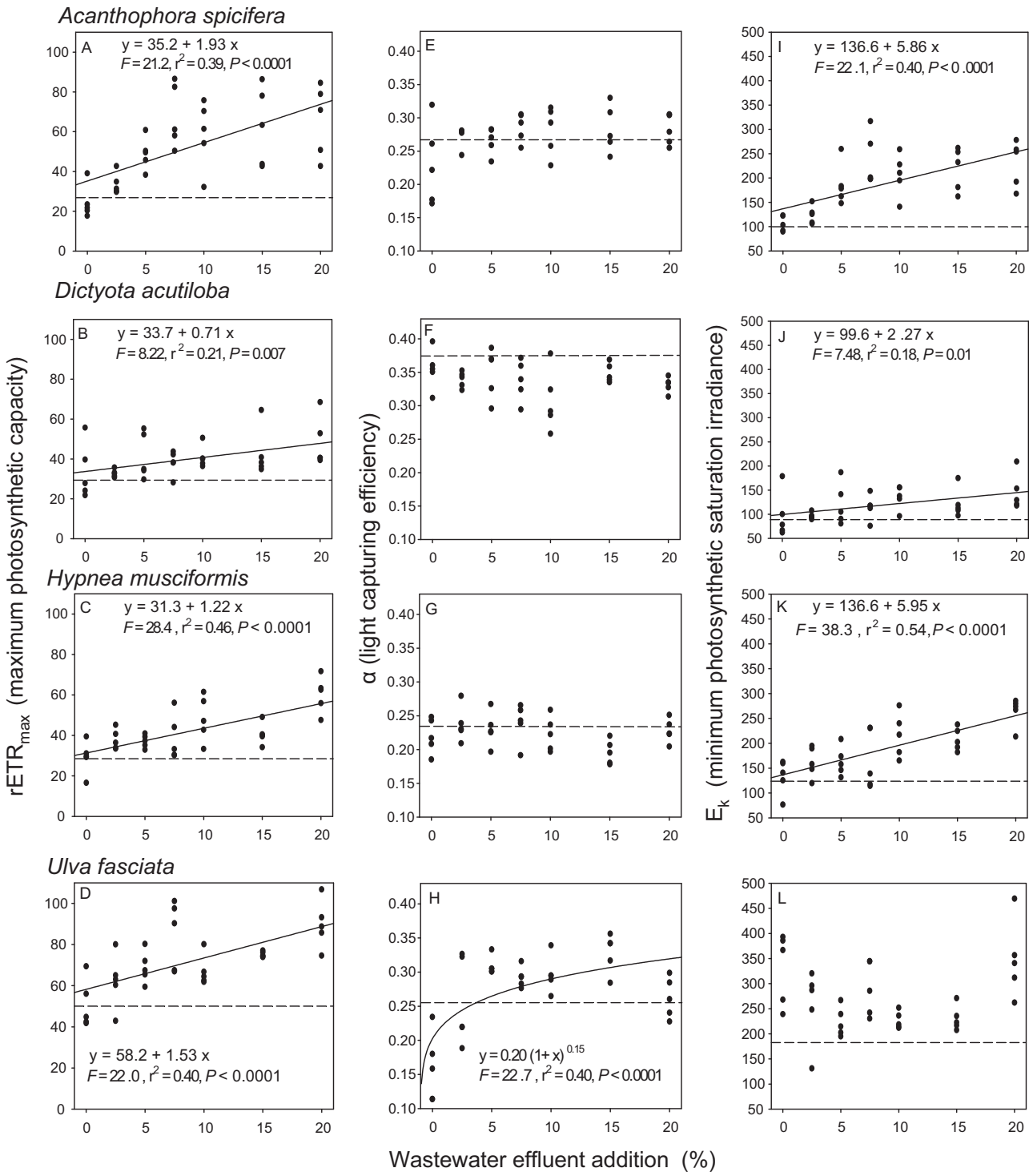

Fig. 5. Final photosynthetic parameters $rETR_{max}$ (a–d), α (e–h) and E_k (i–l) for *Acanthophora spicifera*, *Dictyota acutiloba*, *Hypnea musciformis*, and *Ulva lactuca* in response to increasing wastewater effluent addition (%); dashed lines represent average initial values.

(*H. musciformis* trial only), iron (Fe), and manganese (Mn) with increasing additions of wastewater effluent (Tables 4 and 5). No significant difference was found between the no addition and additions of wastewater effluent in day 8 nutrient concentrations of copper (Cu), molybdenum (Mo), and zinc (Zn) (Tables 4 and 5). In the *U. lactuca* trial, TP was only detected in the two highest treatments, Cu was detected in 4 treatments, and Mo and Zn were not detected in any treatment (Table 5).

Significant differences in final wet weights of *Acanthophora spicifera* and *Dictyota acutiloba* were found between the no addition and wastewater effluent additions of 2.5–10.0% and 5.0–20.0%, respectively (Table 3). Significant differences were found in the final wet weights of *Hypnea musciformis* and *Ulva lactuca* between the no addition and all wastewater effluent additions (Tables 4 and 5). Furthermore, the final wet weights of *H. musciformis* and *U. lactuca* in the no addition (0.56 ± 0.03 and 0.56 ± 0.01, respectively) were 2-fold lower than those provided with the lowest wastewater effluent addition of 2.5% (1.19 ± 0.05 and 1.10 ± 0.03, respectively) (Tables 4 and 5). The highest final wet weights of *A. spicifera* (0.85 ± 0.08), *D. acutiloba* (0.78 ± 0.03), *H. musciformis* (1.73 ± 0.07),

Table 3
Final wet weight (ww), day 8 nutrient concentrations, nutrient uptake rates ($g^{-1}\,d^{-1}$) (means ± SE), and % change in nutrient concentration from day 8 to 9 per treatment for *Acanthophora spicifera* and *Dictyota acutiloba*. Significant differences between the no addition and wastewater effluent additions are in bold, n.d. = not detected.

	No addition	2.5%	5.0%	7.5%	10.0%	15.0%	20.0%
Acanthophora spicifera							
Final ww (g)	0.42 ± 0.01	**0.77 ± 0.02***	**0.70 ± 0.03****	**0.78 ± 0.06****	**0.85 ± 0.08***	0.63 ± 0.06	0.77 ± 0.03
Total organic carbon (TOC)							
Day 8 (μM)	128 ± 11	99.5 ± 6.9	90.8 ± 2.6	112 ± 7.6	190 ± 41	126 ± 3.7	157 ± 2.7
TOC uptake ($\mu M\,g^{-1}\,d^{-1}$)	−0.71 ± 40	−25.0 ± 16	−25.6 ± 3.7	−44.8 ± 6.6	16.1 ± 16	−45.7 ± 17	−24.3 ± 15
% Change	−6.67	−24.2	−20.8	−33.4	−0.24	−24.1	−11.3
Total nitrogen (TN)							
Day 8 (μM)	8.6 ± 0.2	**12.8 ± 0.5***	**17.1 ± 0.5****	**24.8 ± 0.9****	**30.3 ± 1.8****	**45.4 ± 1.1*****	**57.3 ± 0.8*****
TN uptake ($\mu M\,g^{-1}\,d^{-1}$)	−2.2 ± 1.2	2.3 ± 0.8	**7.9 ± 0.5***	4.8 ± 2.5	**15.5 ± 0.3****	**36.9 ± 2.4****	**32.5 ± 1.7*****
% Change	−13.0	13.9	32.1	15.3	44.7	51.1	43.6
Nitrate (NO_3^-)							
Day 8 (μM)	0.27 ± 0.11	**4.5 ± 0.03*****	**10.3 ± 0.3*****	**15.1 ± 0.2*****	**18.0 ± 1.0*****	**32.4 ± 0.4*****	**41.3 ± 0.2*****
NO_3^- uptake ($\mu M\,g^{-1}\,d^{-1}$)	0.21 ± 0.07	**5.6 ± 0.1*****	**14.7 ± 0.4*****	**19.1 ± 0.4*****	**20.8 ± 1.1*****	**42.4 ± 3.9****	**31.8 ± 0.8*****
% Change	57.8	94.3	98.7	98.7	98.9	81.6	59.4
Dictyota acutiloba							
Final ww (g)	0.53 ± 0.02	0.53 ± 0.01	**0.67 ± 0.01***	**0.71 ± 0.02***	**0.64 ± 0.02***	**0.72 ± 0.01***	**0.78 ± 0.03*****
Total organic carbon (TOC)							
Day 8 (μM)	111 ± 10	128 ± 8.8	162 ± 37	150 ± 9.6	254 ± 45	149 ± 47	173 ± 9.2
TOC uptake ($\mu M\,g^{-1}\,d^{-1}$)	−185 ± 31	−90.1 ± 28	−148 ± 111	−157 ± 68	**178 ± 64***	25.6 ± 38	**−14.2 ± 14***
% Change	−86.9	−41.6	−110	−66.2	30.4	3.64	−7.81
Total nitrogen (TN)							
Day 8 (μM)	10.8 ± 1.2	13.2 ± 0.5	27.6 ± 3.6	31.7 ± 5.0	**33.4 ± 2.4****	**54.2 ± 2.0*****	**60.2 ± 1.4*****
TN uptake ($\mu M\,g^{-1}\,d^{-1}$)	−1.97 ± 2.8	6.6 ± 2.8	16.4 ± 5.7	**17.7 ± 0.5***	19.2 ± 8.0	**56.2 ± 4.2****	**58.9 ± 1.6*****
% Change	−21.0	28.5	32.9	45.0	42.2	76.1	76.1
Nitrate (NO_3^-)							
Day 8 (μM)	0.35 ± 0.03	**4.2 ± 0.3****	**8.1 ± 0.5*****	**14.9 ± 0.5*****	**19.6 ± 0.9*****	**32.7 ± 0.7*****	**40.5 ± 0.6*****
NO_3^- uptake ($\mu M\,g^{-1}\,d^{-1}$)	0.09 ± 0.03	**6.8 ± 1.2***	**11.9 ± 0.5****	**20.8 ± 0.8*****	**29.5 ± 0.9*****	**45.1 ± 1.2*****	**50.6 ± 0.9*****
% Change	42.2	80.0	97.8	98.0	96.8	99.4	97.0
Nitrite (NO_2^-)							
Day 8 (μM)	n.d.	**0.47 ± 0.05****	**0.90 ± 0.06****	**1.72 ± 0.09*****	**2.2 ± 0.1*****	**3.9 ± 0.1*****	**4.8 ± 0.1*****
NO_2^- uptake ($\mu M\,g^{-1}\,d^{-1}$)	–	**0.89 ± 0.09****	**1.35 ± 0.10*****	**2.4 ± 0.1*****	**3.4 ± 0.2*****	**5.3 ± 0.1*****	**4.4 ± 0.5*****
% Change	–	100	100	100	100	100	100

* $P < 0.05$.
** $P < 0.005$.
*** $P < 0.0005$.

and *U. lactuca* (1.40 ± 0.11) were observed in treatments of 10.0–20.0% (Tables 3–5).

TN uptake rates of *Acanthophora spicifera*, *Hypnea musciformis*, and *Ulva lactuca* in treatments ≥5.0% wastewater effluent were significantly increased from those of the no addition (Tables 3–5). The highest TN uptake rates (μmol TN $g^{-1}\,d^{-1}$) of *A. spicifera* (36.9 ± 2.40), *Dictyota acutiloba* (58.9 ± 1.56), *H. musciformis* (28.1 ± 3.84), and *U. lactuca* (31.4 ± 3.44) were observed in wastewater effluent additions of 15.0–20.0% (Tables 3–5). The % change in TN for all species was lower than 100%. NO_3^- uptake rates of all species in treatments ≥2.5% wastewater effluent were significantly increased from those of the no addition and *U. lactuca* removed 100% of NO_3^- in all treatments (Tables 3–5). The highest NO_3^- uptake rates (μmol NO_3^- $g^{-1}\,d^{-1}$) of *A. spicifera* (42.4 ± 3.90), *D. acutiloba* (50.6 ± 0.91), *H. musciformis* (24.9 ± 2.81), and *U. lactuca* (28.5 ± 0.21) occurred in wastewater effluent additions of 15.0–20.0% (Tables 3–5). *H. musciformis* removed 100% of TP, Fe, Mo, and Zn in all treatments (with the exception of Fe in the 10.0% wastewater effluent addition and Mo in the no addition) (Table 4). The highest Mn uptake rates of *H. musciformis* (5.79 ± 0.93 ppb $g^{-1}\,d^{-1}$) and *U. lactuca* (2.32 ± 0.73 ppb $g^{-1}\,d^{-1}$) occurred in wastewater effluent additions of 20.0% and 7.5%, respectively (Tables 4 and 5). Mn uptake rates of *H. musciformis* in wastewater effluent additions of 7.5, 10.0, and 20.0% were significantly higher than those of the no addition (Table 4). For all species, in all treatments, the total organic carbon concentrations of the incubation water generally increased from day 8 to 9 (Tables 3–5).

3.2.4. % tissue N and $\delta^{15}N$ values of *Hypnea musciformis* and *Ulva lactuca*

The low N acclimated % tissue N of *Hypnea musciformis* (1.02 ± 0.03) and *Ulva lactuca* (1.14 ± 0.29) were significantly ($P < 0.0001$) decreased from bloom levels (2.81 ± 0.70 and 3.31 ± 0.31, respectively). The final % tissue N of *H. musciformis* and *U. lactuca* significantly increased with increasing % wastewater effluent addition (Fig. 6) and concentrations of TN and NO_3^- (Table 2). The $\delta^{15}N$ values of *H. musciformis* and *U. lactuca* after low N acclimation and in the no addition were not significantly changed from initial bloom levels. The final $\delta^{15}N$ values of *H. musciformis* and *U. lactuca* significantly increased with increasing % wastewater effluent addition (Fig. 6) and concentrations of TN and NO_3^- (Table 2). The highest $\delta^{15}N$ values observed were those of *U. lactuca* (30.3 ± 0.3 ‰) in the highest wastewater effluent addition.

4. Discussion

Opportunistic macroalgae in the genus *Ulva* are known to form blooms in response to excess anthropogenic nutrients worldwide (Briand, 1989; Thom and Albright, 1990; Sfriso et al., 1993; Raffaelli et al., 1998; Menendez and Comin, 2000; Hu et al., 2010). Blooms of *Hypnea musciformis* have been documented in nutrient enriched areas of Florida (Avery, 1997; Lapointe and Bedford, 2007), but nutrient driven blooms consisting of both *H. musciformis* and *U. lactuca* have not been documented. This study shows that *H. musciformis* is an opportunistic macroalga capable of physiologically responding to excess nutrients at rates equal to the world renowned *Ulva* spp. in nearshore regions of Maui that are affected by anthropogenic nutrient enrichment.

4.1. N storage of *Hypnea musciformis*

The preliminary N and P experiment on *H. musciformis* confirmed that this species responds to high N conditions by

Table 4
Final wet weight (ww), day 8 nutrient concentrations, nutrient uptake rates ($g^{-1}\,d^{-1}$) (means ± SE), and % change in nutrient concentrations from day 8 to 9 per treatment for *Hypnea musciformis*. Significant differences between the no addition and wastewater effluent additions are in bold, n.d. = not detected.

	No addition	2.5%	5.0%	7.5%	10.0%	15.0%	20.0%
Final ww (g)	0.56 ± 0.03	**1.19 ± 0.05*****	**1.41 ± 0.08*****	**1.38 ± 0.07*****	**1.73 ± 0.07*****	**1.54 ± 0.08*****	**1.54 ± 0.11*****
Total organic carbon (TOC)							
Day 8 (μM)	69.8 ± 4.7	77.1 ± 1.2	73.8 ± 4.4	88.6 ± 3.6	**103 ± 1.0***	104 ± 21	**119 ± 4.9***
TOC uptake ($\mu M\,g^{-1}\,d^{-1}$)	−40.5 ± 13	−13.9 ± 0.9	−24.5 ± 5.5	−24.0 ± 2.3	−7.34 ± 2.7	−16.4 ± 6.5	−22.1 ± 6.2
% Change	−37.5	−21.6	−49.6	−36.8	−12.6	−28.7	−30.3
Total nitrogen (TN)							
Day 8 (μM)	5.9 ± 0.1	**17.0 ± 0.6*****	**29.3 ± 2.8****	**39.8 ± 2.3*****	**54.7 ± 2.0*****	**67.7 ± 17****	**87.0 ± 4.0*****
TN uptake ($\mu M\,g^{-1}\,d^{-1}$)	2.7 ± 0.2	**9.3 ± 0.3*****	**15.5 ± 2.0***	**23.0 ± 1.4****	**26.9 ± 1.1*****	**28.1 ± 3.8***	**25.7 ± 1.1*****
% Change	25.6	65.0	73.4	79.6	85.2	62.0	45.6
Nitrate (NO_3^-)							
Day 8 (μM)	0.19 ± 0.08	**5.4 ± 0.3*****	**9.1 ± 1.2***	**16.0 ± 1.0*****	**22.0 ± 1.0*****	**28.1 ± 7.2****	**44.5 ± 4.9****
NO_3^- uptake ($\mu M\,g^{-1}\,d^{-1}$)	−0.08 ± 0.12	**4.3 ± 0.3****	**6.1 ± 0.9***	**11.5 ± 0.8****	**12.4 ± 0.5*****	**16.4 ± 1.5****	**24.9 ± 2.8****
% Change	21.5	93.4	93.6	98.3	97.8	89.6	85.7
Nitrite (NO_2^-)							
Day 8 (μM)	n.d.	**7.2 ± 0.1*****	**12.6 ± 0.7*****	**19.6 ± 0.6*****	**31.0 ± 1.3*****	**40.8 ± 14****	**54.7 ± 2.2*****
NO_2^- uptake ($\mu M\,g^{-1}\,d^{-1}$)	–	**6.1 ± 0.04*****	**8.9 ± 0.5*****	**14.0 ± 0.5*****	**17.0 ± 0.7*****	**13.2 ± 3.3*****	**11.7 ± 0.3*****
% Change	–	100	99.7	98.3	94.7	43.4	33.2
Ammonium (NH_4^+)							
Day 8 (μM)	n.d.	0.90 ± 0.21	**1.6 ± 0.3***	**2.8 ± 0.4***	**5.9 ± 0.17*****	**8.1 ± 0.51*****	**7.8 ± 1.1***
NH_4^+ uptake ($\mu M\,g^{-1}\,d^{-1}$)	–	0.75 ± 0.17	**1.2 ± 0.2***	**2.0 ± 0.3***	**3.4 ± 0.1*****	**5.3 ± 0.3*****	**5.1 ± 0.7***
% Change	–	100	100	100	100	100	100
Total phosphorous (TP)							
Day 8 (μM)	0.29 ± 0.06	0.52 ± 0.06	**0.81 ± 0.04****	**1.0 ± 0.1****	**1.7 ± 0.04*****	**2.1 ± 0.3*****	**2.6 ± 0.1*****
TP uptake ($\mu M\,g^{-1}\,d^{-1}$)	0.34 ± 0.11	0.44 ± 0.05	0.58 ± 0.03	0.75 ± 0.04	**0.95 ± 0.02***	**1.3 ± 0.1***	**1.8 ± 0.03****
% Change	100	100	100	100	100	100	100
Copper (Cu)							
Day 8 (ppb)	0.99	2.2 ± 0.2	2.1 ± 0.2	1.86	1.7 ± 0.6	2.6 ± 1.7	**3.1 ± 0.3***
Cu uptake ($ppb\,g^{-1}\,d^{-1}$)	0.77	0.45 ± 0.05	−0.41 ± 0.09	0.04	0.41 ± 0.26	1.0 ± 0.54	0.70 ± 0.01
% Change	26.5	21.4	−31.3	2.59	39.0	46.3	33.7
Iron (Fe)							
Day 8 (ppb)	n.d.	**1.2 ± 0.1*****	1.68	**1.3 ± 0.1****	**1.7 ± 0.1****	**1.6 ± 0.4****	2.0 ± 0.06
Fe uptake ($ppb\,g^{-1}\,d^{-1}$)	–	**1.0 ± 0.04*****	1.19	**0.97 ± 0.07****	0.59 ± 0.18	**1.0 ± 0.1****	**1.3 ± 0.04*****
% Change	–	100	100	100	54.0	100	100
Manganese (Mn)							
Day 8 (ppb)	0.39 ± 0.06	**3.6 ± 0.5***	**5.4 ± 0.8***	**6.6 ± 0.2*****	**12.3 ± 0.2*****	**16.0 ± 3.2****	**22.8 ± 0.9*****
Mn uptake ($ppb\,g^{-1}\,d^{-1}$)	0.69 ± 0.11	2.7 ± 0.5	3.0 ± 0.6	**3.1 ± 0.4***	**5.5 ± 0.2*****	2.8 ± 1.2	**5.8 ± 0.9***
% Change	100	85.9	73.1	65.2	78.1	23.5	38.2
Molybdenum (Mo)							
Day 8 (ppb)	6.4 ± 0.2	**8.9 ± 0.3****	7.0 ± 0.5	6.3 ± 0.1	7.2 ± 0.3	5.4 ± 0.3	7.3 ± 0.3
Mo uptake ($ppb\,g^{-1}\,d^{-1}$)	9.8 ± 0.9	7.4 ± 0.2	**5.0 ± 0.4***	**4.6 ± 0.04***	**4.2 ± 0.2***	**3.5 ± 0.2***	**4.7 ± 0.2***
% Change	85.7	100	100	100	100	100	100
Zinc (Zn)							
Day 8 (ppb)	7.4 ± 3.7	5.1 ± 1.4	1.6 ± 0.2	5.0 ± 1.0	5.8 ± 1.1	8.9 ± 1.0	**7.9 ± 0.9*****
Zn uptake ($ppb\,g^{-1}\,d^{-1}$)	13.2 ± 6.6	4.3 ± 1.2	1.2 ± 0.1	3.6 ± 1.2	3.4 ± 0.7	5.8 ± 0.7	5.2 ± 0.6
% Change	100	100	100	100	100	100	100

* $P < 0.05$.
** $P < 0.005$.
*** $P < 0.0005$.

forming dark purple phycobilin pigments (PE and PC) and acclimates to low N conditions by utilizing PE and PC, subsequently lightening in color. Utilization of PE and PC in low N conditions temporarily allowed for sustained growth and photosynthetic rates similar to samples provided with N additions. These findings are comparable to the increased PE and PC levels observed of *Ahnfeltiopsis concinna* in acclimation to low from high light conditions (Beach et al., 2000). In agreement with several studies, these results document the ability of macroalgae to store N which can be used to temporarily maintain growth rates in low N conditions (Lapointe and Tenore, 1981; Björnsäter and Wheeler, 1990; Fong et al., 1998). Therefore, without a low N acclimation period prior to nutrient enrichment experiments conducted on *H. musciformis*, growth rates of samples in the no addition will be reflective of this species utilizing N stores for growth (as those reported by Vermeij et al., 2009).

4.2. Nutrient effects on growth rates

All species visually responded to increasing additions of wastewater effluent (and increased N supply) with darkened coloration. These observations agree with those of Lapointe et al. (1976), where *Hypnea musciformis* responded to increased wastewater N supplies (140 mg/l) with dark brown, almost black pigmentation. All species also increased growth rates when provided with additional N, however, the bloom forming species, *H. musciformis* and *Ulva lactuca*, were more responsive in terms of building biomass than *Acanthophora spicifera* (another bloom forming species) and *Dictyota acutiloba* (non-bloom forming). Although *A. spicifera* is known to form blooms in Hawai'i (Smith et al., 2002) and responded to increased nutrients with increased growth, it was not as responsive as *H. musciformis* and *U. lactuca*. This may be because of the morphological differences between the species, as *A. spicifera* is polysiphonous, *H. musciformis* is *pseudoparenchymatous* and *U. lactuca* is distromatic. *H. musciformis* and *U. lactuca* responded to the increased nutrient levels in the smallest wastewater effluent addition of 2.5% with 2-fold higher biomass than those in the no addition. In addition, the growth rate of *H. musciformis* depleted of N stores and then subjected to the no addition was nearly 3-fold lower than that of the no addition in the preliminary experiment and 5-fold lower than treatments of 10.0% wastewater effluent and above. The maximum growth rates of *H.*

Table 5
Final wet weight (ww), day 8 nutrient concentrations, nutrient uptake rates ($g^{-1}\,d^{-1}$) (means ± SE), and % change in nutrient concentrations from day 8 to 9 per treatment for *Ulva lactuca*. Significant differences between the no addition and wastewater effluent additions are in bold. n.d. = not detected.

	No addition	2.5%	5.0%	7.5%	10.0%	15.0%	20.0%
Final ww (g)	0.56 ± 0.01	**1.10 ± 0.03****	**1.27 ± 0.04*****	**1.16 ± 0.04*****	**1.19 ± 0.11*****	**1.31 ± 0.01*****	**1.40 ± 0.11*****
Total organic carbon (TOC)							
Day 8 (μM)	71.1 ± 2.8	104 ± 15	94.6 ± 9.9	138 ± 24	105 ± 13	69.7 ± 9.4	102 ± 7.3
TOC uptake ($\mu M\,g^{-1}\,d^{-1}$)	−73.1 ± 27	27.4 ± 13	−37.4 ± 9.6	28.3 ± 16	−1.49 ± 6.3	−74.1 ± 13	−62.7 ± 22
% Change	−57.2	17.8	−48.5	8.90	−6.41	−159	−104
Total nitrogen (TN)							
Day 8 (μM)	5.6 ± 0.7	10.9 ± 1.1	**22.5 ± 0.6*****	**38.1 ± 5.4***	**35.0 ± 2.7****	**40.7 ± 5.4***	**58.9 ± 4.7****
TN uptake ($\mu M\,g^{-1}\,d^{-1}$)	0.43 ± 1.9	5.1 ± 0.8	**11.7 ± 0.4***	**26.7 ± 4.5***	**21.9 ± 1.4*****	**21.4 ± 2.6***	**31.4 ± 3.4***
% Change	5.35	49.5	66.3	79.8	74.9	69.1	73.6
Nitrate (NO_3^-)							
Day 8 (μM)	0.05 ± 0.03	**4.8 ± 0.3***	**11.2 ± 1.5***	**25.2 ± 2.6****	**23.7 ± 0.4*****	**31.3 ± 2.8****	**39.9 ± 0.3*****
NO_3^- uptake ($\mu M\,g^{-1}\,d^{-1}$)	0.27	**4.4 ± 0.3*****	**8.8 ± 1.2***	**21.8 ± 2.2****	**19.9 ± 0.3*****	**23.6 ± 2.0****	**28.5 ± 0.2*****
% Change	100	100	100	100	100	99.0	100
Nitrite (NO_2^-)							
Day 8 (μM)	0.02 ± 0.01	**0.19 ± 0.01****	**0.62 ± 0.01*****	**0.85 ± 0.07****	**0.98 ± 0.07****	**1.08 ± 0.14***	**1.54 ± 0.13****
NO_2^- uptake ($\mu M\,g^{-1}\,d^{-1}$)	0.10	**0.17 ± 0.01***	**0.49 ± 0.01*****	**0.74 ± 0.06****	**0.82 ± 0.06****	**0.83 ± 0.10***	**1.10 ± 0.09****
% Change	100	100	100	100	100	100	100
Ammonium (NH_4^+)							
Day 8 (μM)	1.59 ± 0.11	n.d	1.67	3.75	1.72	0.82	0.57
NH_4^+ uptake ($\mu M\,g^{-1}\,d^{-1}$)	2.8 ± 0.20	–	1.31	3.23	1.45	0.63	0.41
% Change	100	–	100	100	100	100	100
Total Phosphorous (TP)							
Day 8 (μM)	n.d	n.d	n.d	n.d	n.d	0.05	0.13 ± 0.06
TP uptake ($\mu M\,g^{-1}\,d^{-1}$)	–	–	–	–	–	0.03	0.09 ± 0.04
% Change	–	–	–	–	–	100	100
Copper (Cu)							
Day 8 (ppb)	2.7 ± 0.3	1.28 ± 0.37	2.4 ± 0.2	3.0 ± 0.8	1.66 ± 0.21	1.33 ± 0.39	3.4 ± 0.3
Cu uptake ($ppb\,g^{-1}\,d^{-1}$)	0.20 ± 0.89	−1.28 ± 0.81	0.66 ± 0.54	0.79 ± 0.78	−1.08 ± 0.11	−1.24 ± 0.15	0.13 ± 0.14
% Change	−6.26	−74.0	21.3	−6.09	−84.7	−83.5	2.98
Iron (Fe)							
Day 8 (ppb)	1.89	n.d	n.d	1.20 ± 0.17	1.54 ± 0.34	2.2	2.5 ± 0.4
Fe uptake ($ppb\,g^{-1}\,d^{-1}$)	3.4	–	–	0.12 ± 0.33	1.30 ± 0.29	1.67	1.75 ± 0.25
% Change	100	–	–	26.4	100	100	100
Manganese (Mn)							
Day 8 (ppb)	1.09 ± 0.05	2.4 ± 0.3	**7.5 ± 0.2*****	**12.1 ± 0.8****	**15.5 ± 1.2****	**22.4 ± 2.1****	**32.5 ± 2.5****
Mn uptake ($ppb\,g^{-1}\,d^{-1}$)	1.05 ± 0.44	−0.14 ± 0.34	0.54 ± 0.32	1.44 ± 1.35	2.32 ± 0.73	0.22 ± 1.3	2.2 ± 1.7
% Change	59.8	−10.3	8.38	9.72	16.1	−2.42	6.95

* $P < 0.05$.
** $P < 0.005$.
*** $P < 0.0005$.

musciformis and *U. lactuca* in this study were approximately 0.24 g d^{-1}, which is slightly slower than 0.34 g d^{-1} reported for *U. lactuca* in the Roskilde Fjord (Pedersen and Borum, 1996). However, in the summer months, these northern fjords receive 16 h of sunlight (Pedersen and Borum, 1996) compared to 12 h in Hawai'i. These results are similar to those of Larned (1998) where samples of *U. lactuca* provided with additions of N and NP grew significantly faster than those in the control or P addition, suggesting that *U. lactuca* is N limited in Hawai'i. The high growth rates of *H. musciformis* and *U. lactuca* (~15% d^{-1}) are comparable to those observed for the bloom forming *U. lactuca* in Waquoit Bay from *in situ* N and P enrichment experiments (over 13 d) (Teichberg et al., 2008). Our findings are also consistent with those of other studies on *Ulva* that report increased growth rates with increased nutrient supplies (Björnsäter and Wheeler, 1990; Pedersen and Borum, 1996, 1997; Fox et al., 2008).

4.3. Nutrient effects on photosynthetic properties

All bloom forming species tested in this study responded to increased nutrients with increased photosynthetic performance ($rETR_{max}$) and saturation irradiance (E_k). These findings are consistent with those of Valiela et al. (1997) where elevated maximum photosynthetic rates were characteristic of bloom forming macroalgae in increased nutrient conditions. These results also agree with those of Pedersen and Borum (1996) who found that out of 12 species tested in laboratory N enrichment experiments, *U. lactuca* had the highest growth rates and suffered the most from N limitation. In this study, *U. lactuca* was the only species to show substantial declines in photosynthetic properties when deprived of nutrients. The photosynthetic efficiency (α) and % tissue N of *U. lactuca* were significantly decreased after a total of 16 days in low N conditions (seven days of low N acclimation followed by nine days in the no addition). These results (1) support the findings of Rosenberg and Ramus (1982) which conclude that N pools in *Ulva* consist mainly of chlorophyll-protein complexes, (2) agree with those of Henley et al. (1991) (for *U. rotundata* over seven days) that the light capturing efficiency of *U. lactuca* is negatively affected by low N conditions, and (3) verify that chlorophyll–protein complexes are utilized in *U. lactuca* when subjected to low N conditions for extended periods of time.

4.4. Nutrient uptake rates and total organic carbon release

All species were able to take up high levels of TN, NO_3^-, and NO_2^- (with the exception of the *A. spicifera* trial where NO_2^- was not detected) and uptake rates increased with increasing wastewater effluent additions. These results are similar to those of other studies where bloom forming macroalgae increased nutrient uptake rates in response to increased nutrient supplies (Peckol et al., 1994; Pedersen and Borum, 1997; Valiela et al., 1997). The % change in TN for all species was lower than 100%, indicating that the samples were not likely N-limited. The TN uptake rates observed in this study are comparable to those

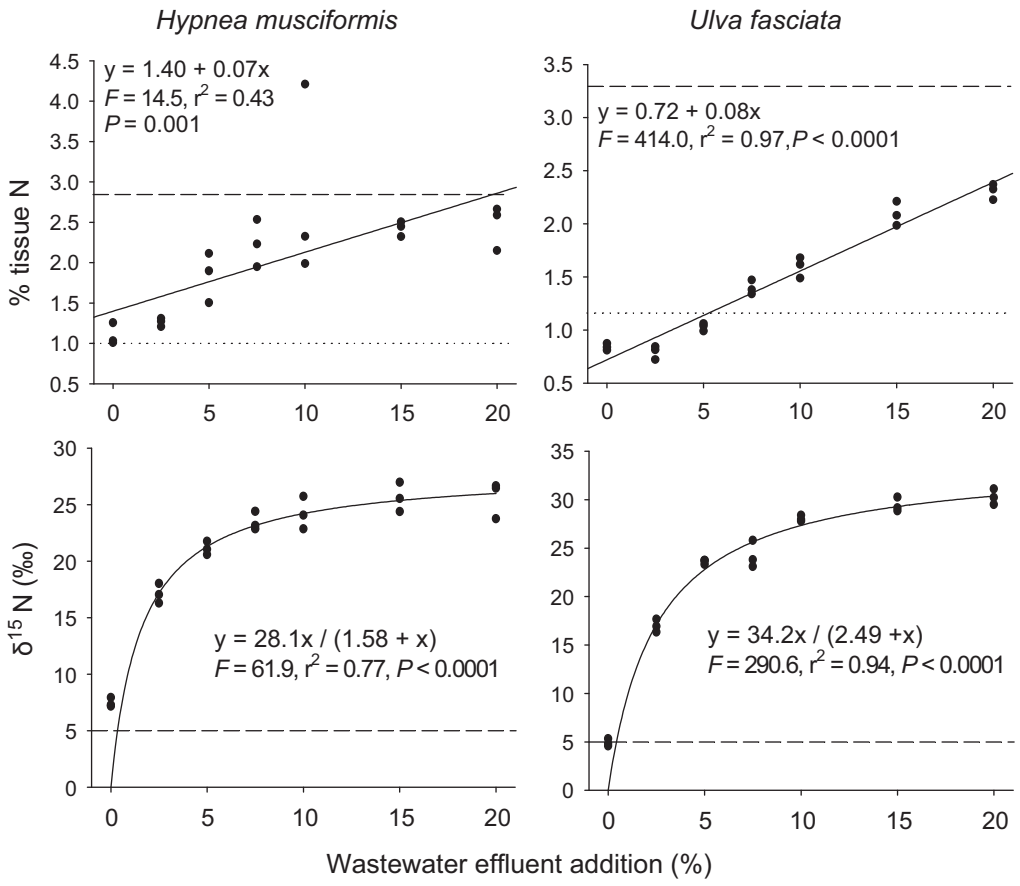

Fig. 6. Final % tissue N and $\delta^{15}N$ values of *Hypnea musciformis* and *Ulva lactuca* in response to increasing wastewater effluent addition (%). Average field (bloom) and low N acclimated levels are represented by dashed and dotted lines, respectively (no change occurred in $\delta^{15}N$ values during the low N acclimation period).

reported by Björnsäter and Wheeler (1990) for *U. fenestrata* under N-enriched and P-limited conditions (27.1 ± 6.9 μmol TN g^{-1} d^{-1}), which were significantly lower than those in the N and P enriched treatment (133.7 ± 9.3 μmol TN g^{-1} d^{-1}). Björnsäter and Wheeler (1990) suggested from these findings that algae regulate N and P uptake to maintain a balanced internal N:P ratio, which indicates that the species tested in this study might have higher N uptakes rates when provided with higher P concentrations. *H. musciformis* and *U. lactuca* acquired Mn and all available Fe from the surrounding media. *H. musciformis* also acquired all available P, Mo, and Zn confirming that micro nutrients such as Fe, Mn, Mo, and Zn are vital for growth and photosynthetic properties.

H. musciformis NO_3^- uptake might have been affected by limited NO_3^- reductase activity because of the presence of NH_4^+ (Thomas and Harrison, 1985; Young et al., 2005). Another, more likely, possibility is that NO_3^- uptake was limited by Fe because NO_3^- utilization depends on Fe-containing enzymes and requires high cellular Fe quotes (Viaroli et al., 2005). In the highest wastewater effluent addition, all available Fe was utilized but the nitrate was only decreased by 85%. However, Maui soils are derived from intermediate-weathering of basalt and are rich in Fe and other nutrients (Chorover et al., 2004); therefore it is unlikely that the blooms of *H. musciformis* and *U. lactuca* are Fe limited.

For all species, in all treatments, the total organic carbon of the incubation water generally increased over 24 h, which is consistent with the observations of Khailov and Burlakava (1969) that macroalgae release large amounts of organic matter. Generally, macroalgae frequently fix carbon in excess of metabolic needs for growth and subsequently release the unused dissolved organic carbon (DOC) (Alber and Valiela, 1994). In bloom situations, this is likely to further alter ecosystem properties as substantial quantities of DOC enter the microbial food web (Alber and Valiela, 1994). Furthermore, Smith et al. (2006) document that the dissolved organic matter released from macroalgae promotes microbial growth and subsequently causes coral mortality. The vast majority of coral disease incidences occur on reefs with moderate to high anthropogenic impacts (Green and Bruckner, 2000). DOC loading caused significant coral mortality and increased (by an order of magnitude) growth rates of microbes associated with coral mucus (Kline et al., 2006), which suggests that these microbial assemblages are carbon limited. These findings in the combination with other studies (Pantos et al., 2003; Sutherland et al., 2004) further indicate that increased levels of DOC in wastewater (and other organic sources) from coastal developments could contribute to the high incidence of coral disease on adjacent reefs. Future work on Maui should therefore include efforts in bloom and non-bloom areas to identify microbial communities in terms of composition and abundance in disturbed and natural states.

4.5. % tissue N and $\delta^{15}N$ values

The % tissue N of *Hypnea musciformis* and *Ulva lactuca* decreased from bloom levels when acclimated to low N conditions, then significantly increased with increasing wastewater addition and concentrations of TN and NO_3^-. These results are similar to those of Lapointe et al. (1976) where *H. musciformis* increased % tissue N in high N (3.4%; 140 mg/l) compared to low N (2.5%; 49 mg/l) conditions. In agreement with other studies, these findings confirm that both species are opportunistic, because they are capable of rapidly building N stores when exposed to increased N supplies

(Björnsäter and Wheeler, 1990; Fong et al., 1994; Pedersen and Borum, 1996; Fong et al., 1998; Cohen and Fong, 2005).

McClelland et al. (1997) found that elevated $\delta^{15}N$ values in groundwater from wastewater infiltration act as a ^{15}N enriched tracer in polluted estuaries. Gartner et al. (2002) confirmed that the method of using macroalgal $\delta^{15}N$ values to trace wastewater dispersal in well-mixed oceanic environments is more sensitive (in terms of detection) than conventional techniques. In Moreton Bay Australia, macroalgal $\delta^{15}N$ values have been used to map the reduced impact of sewage outfalls after wastewater treatment facilities were upgraded (Costanzo et al., 2005). In agreement with these studies (and references therein), this study shows that the tissue $\delta^{15}N$ values of *Hypnea musciformis* and *Ulva lactuca* are reflective of the percent of wastewater N exposure. This demonstrates the potential of using *H. musciformis* and *U. lactuca* in transplantation studies to assess the N source in the marine environment over short time periods. Field studies on Maui have confirmed that the $\delta^{15}N$ values of low N acclimated and transplanted *U. lactuca* samples can be used to map wastewater effluent plumes from underground injection wells emerging onto a nearshore reef (Dailer et al., 2010, 2012).

5. Summary and conclusions

This study confirms that in elevated N conditions, *Hypnea musciformis* stores N by building dark colored phycobilin pigments. This study also confirms that *H. musciformis* and *Ulva lactuca* are opportunistic macroalgae capable of exploiting elevated nutrient levels to rapidly generate substantial amounts of biomass. It is also apparent from this study that *H. musciformis* and *U. lactuca* blooms on Maui will collapse if their resources are depleted. The nutrient enhanced accelerated growth rates of these blooming species suggests, in agreement with numerous other studies from temperate and tropical regions (Littler et al., 1991, 1993; Hunter and Evans, 1995; Fletcher, 1996; Morand and Briand, 1996; Lapointe, 1997; Valiela et al., 1997; Menendez and Comin, 2000; Nelson et al., 2003; Lapointe et al., 2005a,b; Lapointe and Bedford, 2007, 2010; see DeGeorges et al., 2010 for a review), that these blooms will proliferate where land-based nutrients are improperly disposed of. Therefore the interception of land-based nutrients is likely the most effective way for resource managers to diminish bloom formations on Maui. Improved control of land-based nutrients entering the nearshore marine environment could be achieved in various ways, including, reducing nitrogen levels in the injected wastewater effluent, eliminating cesspools in coastal areas, and managing runoff from massive active and inactive agricultural regions. Even secondarily treated wastewater effluent has levels of nitrogen and phosphorus that can drive macroalgal bloom formation in coastal areas with long residence times (US EPA, 1972; DeGeorges, 1990). Cesspools can potentially leech elevated nutrients and bacteria into the adjacent coastal area (US EPA, 1972). Agricultural practices have been known to enrich coastal regions worldwide, including those on Maui, for decades (Soicher and Peterson, 1997; Goreau, 2003). Regulating these and other land-based nutrient sources will greatly improve the health of the remaining coral reefs on Maui and throughout the Hawaiian islands.

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