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Macrophyte Community Response to Nitrogen Loading and Thermal Stressors in Rapidly Flushed Mesocosm Systems

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Abstract

A mesocosm system was developed to simulate estuarine conditions characteristic of short water-residence time ecosystems of the Pacific Coast of North America, and used to evaluate the response of multiple macrophyte metrics to gradients of NO₃ loading and temperature. Replicated experiments found that few responses could be directly attributed to NO₃ loading up to 6 x ambient. Some response metrics exhibited weak relationships with nutrient loading but could not be resolved with available statistical power. While direct nutrient responses were found for some species-specific metrics (e.g. green macroalgal growth and biomass, tissue N%, etc.), many patterns were confounded with temperature. Temperature generally had a larger effect on response metrics than did nutrient load. Experimental macrophyte communities exhibited community shifts consistent with the predicted effects of nutrient loading at 20 °C, but there was no evidence of other eutrophication symptoms (phytoplankton blooms or hypoxia) due to the short system-residence time. The *Z. marina* Nutrient Pollution Index (NPI) tracked the NO₃ gradient at 10 °C, but exhibited no response at 20 °C, which may limit the utility of this metric in areas with marked thermal seasonality. Results suggest that teasing apart the influence of temperature and nutrients on the expression of eutrophication symptoms will require complex multi-stressor experiments and the use of indicators that are sensitive across a broad range of conditions.

Keywords

eutrophication; seagrass; macroalgae; nutrients; temperature; *Zostera marina*; *Zostera japonica*; nutrient pollution index; wasting disease

1. Introduction

Estuaries integrate the biology and geochemistry of terrestrial, riverine and oceanic drivers and respond to many forcing functions at both the land and ocean interface. Human activities have dramatically increased the input of nutrients, especially nitrogen inputs, into many ecosystems (Sobota et al. 2013). Increased nitrogen loading has been directly linked to the proliferation of planktonic and macroalgal blooms at a global scale (Glibert et al., 2005;

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Paerl and Scott 2010), with negative impacts on estuarine ecology, human health and economics (Hoagland et al. 2002; Bricker et al. 2008; Compton et al. 2011).

Healthy seagrass communities are often considered an indicator of low anthropogenic impacts because seagrasses have relatively high light requirements and they thrive under low nutrient conditions. When estuarine macrophyte communities receive anthropogenic nutrient loading, algal species tend to dominate and there is increased competition among primary producers for light. This “nutrient loading hypothesis” (Short et al. 1991; Bricker et al. 2008; Brauer et al. 2012) suggests that under excessive anthropogenic nutrient loads, seagrass systems can be replaced by either macroalgae or phytoplankton dominated systems.

Although this process is conceptually simple, determination of nutrient thresholds that negatively impact seagrass communities has been elusive due to the many factors involved (Valiela et al. 1997; Valiela et al. 2000; Nixon et al. 2001; Nielsen et al. 2004). This is further complicated because residence time influences the nutrient loading rate as well as the export of organic matter (Barrón et al. 2003) and biogeochemical processing rates. Valiela et al. (1997) hypothesized that under longer residence times, eutrophication impacts associated with a given nitrogen load would be more severe than at shorter residence times. Few studies have examined or quantified empirical load-response data within short residence time systems. Additionally, thermal characteristics of the water body likely influence susceptibility to eutrophication. Another potential complication includes colonization by non-native species that may be favored by increased nutrients or altered thermal regimes. Consequently, Cloern (2001) stated that the conceptual model of coastal eutrophication in response to nutrient loading will require addressing how system-specific attributes influence ecosystem response.

The nutrient loading hypothesis as applied to estuarine macrophyte communities was pioneered by researchers in New England (Short et al. 1991; Short et al. 1995; Valiela et al. 1997; Nixon et al. 2001), and supported by both field observations and manipulative mesocosm experiments (Hauxwell and Valiela 2004; Howarth and Marino 2006). However, manipulative experiments have only explored systems with long-residence times (e.g. coastal ponds, secondary and tertiary embayments, etc.). For example, pioneering mesocosm studies (Burkholder et al. 1992; Short et al. 1995; Taylor et al. 1995; Taylor et al. 1999) used long residence times (daily exchange of about ~10–20 %) and warm water temperatures (> 20 °C), which favor development of eutrophic conditions (e.g. organic matter build up, high respiration rates, etc.). These conditions may exacerbate experimental effects and are not necessarily representative of field conditions in open coast estuaries along the Pacific Coast of North America (Hickey and Banas 2003; Lee and Brown 2009), or other upwelling regions such as the Iberian Peninsula, Chilean coast or Benguela Current system (Varela et al. 2005; Pitcher et al. 2010; Barton et al. 2015).

Many estuaries in upwelling-dominated areas may not conform to traditional estuarine paradigms (see Pitcher et al. 2010 for examples). Estuarine systems in the Pacific Northwest (PNW) region of the United States, such as Yaquina Bay, OR, receive large nutrient loads ($2227 \text{ kg N km}^{-2} \text{ y}^{-1}$; Brown and Ozretich 2009) primarily from natural sources and have not exhibited seagrass losses (Kaldy 2014). In contrast to many systems with high nutrient

concentrations, the primary nitrogen source (both riverine and oceanic) is NO_3 coming from either nitrogen fixation by red alder trees in the watershed or coastal upwelling (Lee and Brown 2009; Brown and Ozretich 2009). Further, many estuaries along the Oregon coast are characterized by short residence times on the order of 2–12 days, with a large tidal prism relative to estuary volume, and are considered well mixed systems (Hickey and Banas 2003; Lee and Brown 2009; Lemagie and Lerczak 2015). Likewise, the occurrence of hypoxic water and off-shore phytoplankton blooms are often linked to advection of recently upwelled water masses rather than estuarine eutrophication processes (Brown and Power 2011). Consequently, we wondered if there was a nutrient loading rate that would trigger severe eutrophication expression in systems characterized by short system residence time, since this could be a factor contributing to the moderately low expression of eutrophication observed in many PNW estuaries (Bricker et al. 2007, 2008).

Another factor that has not generally been evaluated in estuarine ecosystems is the response of non-native species to eutrophication and the possible influence on community structure (Cloern 2001). The non-native seagrass *Zostera japonica* has colonized many estuarine systems in the PNW and continues to spread (Shafer et al. 2014). The colonization process transforms mudflat to structured seagrass habitat, although the ecological consequences are unclear. *Z. japonica* colonization has led to control and eradication efforts (Shafer et al. 2014). Recent work concluded that this species is favored in high intertidal habitats with low bathymetric slope and by warm water temperatures (Shafer et al. 2014; Kaldy et al. 2015); however, little is known about the response to nitrogen loading which could influence colonization potential.

Many PNW estuaries have a moderately low expression of eutrophication symptoms (Bricker et al. 2007, 2008) despite high nutrient loads, which appear to contradict predictions of the nutrient loading hypothesis. Consequently, we wanted to determine if there was a nutrient loading threshold that would cause these systems to express more extreme eutrophication impairment. Estuarine plant communities, seagrass, macroalgae and microalgae, were subjected to a factorial experiment with a gradient of NO_3 loading across two temperatures to assess a suite of morphological, physiological and biogeochemical response metrics. We chose nutrient loading rates that represent a realistic gradient of potential anthropogenic nutrient inputs. Likewise, we chose two thermal regimes that mimic conditions near the mouth (ocean dominated) and upstream near the limit of *Z. marina* distribution (riverine dominated). We predicted that PNW estuarine macrophyte communities would exhibit most severe eutrophication symptoms (species shifts, algal blooms, increased production and respiration) under medium or high nitrate loading and warm thermal regimes. To test this hypothesis, we developed a mesocosm system that simulated estuarine conditions of PNW open coast estuaries, which are characterized by large tidal exchange and short residence time.

Research on seagrass ecosystems response to nutrients has focused primarily on simple, species specific metrics such as biomass or growth rates. Here, we use a variety of integrative metrics to assess responses including community structure, the nutrient pollution index, ecosystem metabolism, wasting disease index and shoot mortality. Community structure or dominance is a direct assessment of changes in macrophyte dominance and is

directly related to the nutrient loading hypothesis. Consequently, we expected community structure to strongly reflect responses to mesocosm treatments. The Nutrient Pollution Index (NPI; Lee et al. 2004) is the ratio of leaf nitrogen to leaf mass and has been proposed as a metric to quantify nitrogen availability. Our experiments provide an opportunity to rigorously validate the efficacy of the metric, expecting that the metric would accurately track the NO_3 loading gradient. We also use ecosystem metabolism measured as O_2 dynamics to evaluate community response to NO_3 and thermal stressors. We expected that as species composition shifted and organic matter accumulated there would be an increase in ecosystem respiration. Further, we evaluated the whole plant Wasting Disease Index (WDI; Burdick et al. 1993) because previous work has shown that *Z. marina* subjected to warming and NO_3 loading may be more susceptible to wasting disease. Our expectation was that the WDI would increase with temperature and NO_3 loading. Likewise, since shoot mortality is the ultimate manifestation of integrated response metrics, we examined mortality responses to NO_3 and thermal stress, expecting greater mortality under high NO_3 loads and warm temperatures. Our results indicate that there may be species specific responses to nitrate loading and that temperature and residence time may influence the expression of eutrophication symptoms.

2. Methods

2.1 Mesocosm Conditions

The mesocosm facility (Kaldy 2012; 2014; Shafer et al. 2011) consisted of 8 replicate cabinets (Fig. 1) that contained 2 banks of LED grow lights (180 W Advance Spectrum Max; AIBC International, LLC., Ithaca, NY, USA) suspended over a white polycarbonate tank (60 cm wide x 60 cm deep x 90 cm long). Each LED panel consisted of 119 LED's in a 6:1:1:1 ratio of red (660 nm λ), blue (465 nm λ), orange (620 nm λ) and white (6000 k). Photosynthetic photon flux density (PPFD) of photosynthetically active radiation (400–700 nm λ) measured at the bottom of the tank with a spherical quantum sensor (LI-193SA) and LI-1400 data logger (LI-COR, Lincoln, Nebraska, USA) was $>200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, which is above light saturation for *Z. marina* and *Z. japonica* (Shafer and Kaldy 2014). Mesocosms were maintained on a 12:12 light:dark cycle. Each polycarbonate tank held 324 l and the ambient seawater delivery rate was 500 ml min^{-1} resulting in an exchange rate of about $200 \% \text{ d}^{-1}$. Each cabinet had a separate seawater head tank that eliminated pressure changes and the water delivery rate was controlled using adjustable flowmeters (Model FR4L53BLBN-OM, FL2050; Omega, Stamford, CT, USA). Inflow was checked daily using a graduated cylinder and adjusted as needed. Estuarine tidal cycles were simulated twice daily in each mesocosm using dedicated small submersible pumps (Eco66, Sunlight Supply, Inc., Vancouver, WA., USA) and electronic timers (Apollo 7, Titan Controls, Sunlight Supply, Inc., Vancouver, WA., USA) that were synchronized between tanks. Water was removed by pumping over a 3-h period, such that at simulated low tide the mesocosm volume was reduced by 75%, and then the tank gradually refilled over the next 9 h before the cycle repeated. At “low tide” there was about 4–5 cm of water over-topping the transplant containers. Additionally, each mesocosm had a dedicated submersible pump (Eco66) that was mounted on the wall to maintain mixing within the tank. A preliminary dye study without plants showed complete mixing within a tank took about 2.5 minutes.

Transplanted plant communities were allowed to acclimate in the mesocosms to ambient water temperature and nutrients for 72 h prior to initiation of the manipulative experiments. Raw seawater (unfiltered) was run through the tanks prior to manipulations, which allowed the tanks to be inoculated with a phytoplankton community (see additional details below).

2.2. Macrophyte Collection and Initial Conditions

For each experiment fresh macrophyte material was collected from Yaquina Bay, OR, USA. During low tide, we collected 190 shoots of *Z. marina* and a small bucket of drift green macroalgae (*Ulva* spp.) from the lower portion of the estuary ~ 5 km from estuary mouth. Plants were returned to the lab and held in flowing seawater tables overnight before being cleaned, processed and distributed to mesocosms. *Z. marina* plants were trimmed to consistent dimensions; old leaves were removed at the sheath, and the 3–4 youngest leaves were trimmed to 50 cm length measured from emerging rootlets and 5 rhizome internodes (Kaldy 2014). Epiphytes were removed by wiping both sides of the sheath and each leaf with a wet sponge. Two shoots of *Z. marina* were then transplanted into each of 80 plastic nursery pots (13 cm diameter x 11 cm deep), which were filled with estuarine sand that had been pre-conditioned for 5 days in flowing seawater tanks. Each mesocosm tank contained 10 nursery pots with 20 *Z. marina* shoots. Green macroalgae (GMA) were rinsed with seawater over a 5 mm mesh to remove sediment and debris and picked through to remove invertebrates. Clean GMA was spun in a salad spinner to remove water and 100 g wet weight was added to each mesocosm tank. *Zostera japonica* plants used in this experiment were collected from Yaquina Bay. Since the high shoot densities, small plant size and thin, brittle rhizomes of *Z. japonica* were not conducive to bare root transplant methods (Shafer and Kaldy 2014), a sod of plants and associated sediments were removed with a shovel and placed into a plastic box (32.5 cm x 17.5 cm x 11 cm). One plastic box containing *Z. japonica* shoots was introduced directly to each mesocosm and no effort was made to clean epiphytes or sediment from each individual shoot.

2.3. Experimental Design

We used a randomized factorial design to evaluate the effects of temperature at 10 or 20 °C (cold, warm) and four levels of nutrient loading (ambient, low, medium, high) on estuarine macrophyte communities. To increase statistical power, we ran the 21 d experiment three times during the summer of 2013 (10 June to 1 July; 15 July – 5 August; 9 – 30 September) for total $n = 24$. Temperature treatments were maintained ± 2 °C using 2–3 aquarium heaters (300 W titanium) or EcoPlus ¼ hp aquarium chillers with a Eco633 submersible pump (Sunlight supply Inc., Vancouver, WA USA). All tanks received ambient nutrient loading (sand filtered, ~70 μm) as supplied by the Hatfield Marine Science Center seawater system. Treatment nutrient loading rates (low, medium and high) were nominally calculated as 1.5x, 3x and 6x ambient, respectively. Target loadings were calculated assuming that the long-term median nitrate concentration (11 $\mu\text{M NO}_3$; Lee & Brown 2009) was representative of ambient conditions and that the water turn-over rate in the tank was 200% d^{-1} (720 l d^{-1}). Thus, ambient nutrient load was expected to be about 8 mmols N d^{-1} and the target loading rates of 12, 24 and 50 mmols N d^{-1} corresponding to low, medium and high treatments respectively, were based on these assumptions. To meet these targets, we estimated that we needed to provide an additional 4, 16 and 40 mmols N d^{-1} to the ambient load. Stock NO_3

solutions were made in 18 l batches from ultrapure Milli-Q water mixed with pre-weighed reagent grade KNO_3 (MP Biomedicals LLC., Solon, OH, USA) to give final concentrations of 0.9, 3.7 and 9.7 mmols N l^{-1} for the low, medium and high nutrient treatments respectively. Nitrate additions were made by continuous slow drip of aqueous stock KNO_3 solutions through micro-bore tubing from a header tank into the mesocosm tanks at a rate of $\sim 4 \text{ l d}^{-1}$. The ambient nutrient loading treatment received the same volume of ultrapure Milli-Q water (EMD Millipore, Billerica, MA, USA). Nitrate stock solutions in the header tanks were replenished daily to maintain consistent head pressure and drip rates. Temperature and nutrient drip rates were measured at least twice daily to ensure constant treatment conditions. The water inflow rate was measured daily to ensure that turn-over rates were consistent. At initiation of the manipulative treatments, the seawater source was switched from raw to filtered and the tidal simulation, temperature control and nutrient loading were started.

2.4. Response metrics

Water column dissolved inorganic nutrient (DIN; $\text{NO}_2 + \text{NO}_3$, NH_4 and PO_4); total N and total P and chl *a* concentrations were sampled daily; 300 ml of water was filtered through a 45 mm GFF filter. Aliquots (15 ml) of filtrate were stored frozen for DIN and were subsequently analyzed using Lachat flow injection instrumentation. A 250-ml aliquot of filtrate was transferred to clean brown Nalgene bottles, acidified ($\text{pH} < 2$) with H_2SO_4 and frozen. Samples were analyzed for total dissolved N and P (TDN and TDP respectively) by alkaline persulfate digestion followed by flow injection analysis for NO_3 and PO_4 . Organic N and P were calculated by subtraction (total dissolved minus dissolved inorganic). The filter was placed into a 15-ml centrifuge tube and stored frozen until analyzed for chl *a*. Filters were cold extracted with 90% acetone and analyzed using a calibrated fluorometer (Trilogy, Turner Designs, Sunnyvale, CA USA).

We measured a variety of biological response metrics for the seagrasses and macroalgae. Specifically, we measured final biomass, growth rates, and tissue C:N (thallus, new leaf, old leaf and rhizome+root). *Zostera marina* growth rates were measured as described by Kaldy (2014), while a clip-harvest method was used for *Z. japonica* (Kaldy 2006). GMA growth rate was calculated as the change in biomass (g dw) divided by the length of the experiment in days. For *Z. marina* we also measured sheath width, total leaf length, maximum leaf area index (longest leaf) and new leaf weight per unit area (mg dw cm^{-2}). Using the %N and leaf weight per unit area of new leaf tissue we calculated the “Nutrient Pollution Indicator” proposed by Lee et al. (2004). We specifically targeted newly produced leaves (youngest emergent leaf) since this tissue would most closely reflect experimental conditions. Likewise, we assessed the occurrence of wasting disease following Burdick et al. (1993). At the end of the experiment, we quantified the microalgal flora (MAF) as $\text{mg chl } a \text{ m}^{-2}$ by scraping a known area of the tank wall, transferring the matrix to a GFF filter that was stored frozen until analysis. MAF chl *a* was analyzed as outlined by Janousek and Folger (2012) using a Trilogy fluorometer with the non-acidification module (Turner Designs, Sunnyvale, CA). We assume that the MAF community development is analogous to the epiphyte community and use the term to represent both benthic and epiphyte communities. Main and

McIntire (1974) found no evidence for diatom epiphyte host specificity, implying that our assumption is reasonable.

Community metabolism (dissolved oxygen) was measured at the end of each experiment by turning each mesocosm into a “light/dark bottle” using a modification of the protocol outlined by Spivak et al. (2009). Briefly, at midnight three days before the end of the experiment the inflow and outflow of all tanks were closed off and the tanks were placed in static incubation mode. Temperature and internal mixing were maintained. Mesocosms were bubbled with N₂ gas to reduce oxygen to about 20–50% saturation. A calibrated YSI data sonde was placed in each mesocosm tank and measured temperature, salinity, dissolved O₂ and pH at 5 minute intervals. Each tank was then sealed with a sheet of clear acrylic and large bubbles were removed. Set up of each metabolism measurement was carried out under minimal light conditions (e.g. flashlight). Each tank was incubated in the dark for 5–6 hours to measure community respiration, and ~ 2 hours under full irradiance to measure photosynthetic production. After the incubation period, the acrylic sheet was removed and all flows were restored. Dark respiration (R_d) and gross ecosystem production (GEP) rates were calculated by regression (mg O₂ l⁻¹ versus time) and the ratio of production to respiration (P:R) calculated.

2.5. Statistical Analyses

We used linear mixed effects models to evaluate the effects of nutrients and temperature on biomass, growth rates, tissue C:N, leaf morphology, wasting index, NPI, MAF, and ecosystem metabolism (GP, R_d, P:R). Statistical analyses were performed using the nlme package (Pinheiro et al. 2015) in the R environment (R Core Team 2013). We used the Akaike information criterion (AIC score) to select among four candidate models: nutrient, temperature, additive, and interactive effects. The AIC scores were used to identify factors that affected response variables (determine statistical significance), p-values from specific models were provided to assess potential model uncertainty. Each model controlled for the random effects of tank nested within the three experimental runs. Limited replication (n = 3 per treatment) permitted us to detect treatment effects, but we had low statistical power and were unable to separate variability between replicates from variability between experimental trials. The model with the lowest AIC score, calculated using maximum likelihood (ML) estimation, was selected as the best model (Zuur et al., 2009). The final models were computed using the restricted maximum likelihood (REML) estimation (Zuur et al., 2009). We used a similar statistical structure (linear mixed effects model) that included repeated measures for analysis of daily measurements of water column chl *a*. When a significant nutrient effect was detected, data were analyzed by one-way ANOVA and pairwise comparisons made using Tukey honestly significant difference method. When significant interaction terms were observed in the best model (as chosen by AIC score), data were interpreted based on interaction plots.

3. Results

3.1. Mesocosm conditions

Temperatures were stable throughout the duration of the experiments, fluctuating by no more than 2 °C relative to target values (10 and 20 °C). Nutrient additions resulted in elevated concentrations and loadings (Table 1; Supplemental Figure 1). Actual loading rates were different from the nominal target values due to variability in the estuarine source waters associated with upwelling/downwelling conditions. Likewise, there was variability in average concentrations (Supplemental Figure 1) and loading rates between experiments as a result of variations in estuarine source water. The DIN:P varied between treatments, the medium and high NO₃ loads resulted in water column N:P that could indicate P limitation. The daily mean organic nitrogen (ON) concentrations were similar between treatments and the high DIN:ON ratio at the medium and high loadings means that most of the NO₃ was not being incorporated into biomass (Table 1). This is consistent with the low concentrations (< 0.2 mmol l⁻¹) of dissolved organic carbon measured in all tanks at the end of the experiments (data not shown).

3.2. Microalgal flora

The upper walls of the tanks did not support colonization of MAF, presumably as a result of drying during simulated low tide. The MAF biomass that accumulated on the walls near the bottom of the tanks was variable among experiments and treatments, with mean values ranging between 3.8 ± 2 and 97 ± 35 mg chl *a* m⁻². Mean MAF biomass (46 ± 4.3 mg chl *a* m⁻²) at 10 °C was two-fold higher ($p=0.0097$) than mean MAF biomass (26 ± 2.2 mg chl *a* m⁻²) at 20 °C. No effect of NO₃ loading was detected (Table 2).

3.3. Phytoplankton

Water column chl *a* (WC chl *a*) was used as a proxy for phytoplankton biomass. Visual inspection of plots showing daily WC chl *a* measurements detected temporal patterns that were likely driven by variations in estuarine source water (Fig. 2). Differences between weeks were consistent with in situ fluorescence observed in Yaquina Bay, as well as temporal patterns within each tank. In all of the experiments, WC chl *a* ranged between 0.5 and 12 µg l⁻¹. WC chl *a* varied among the tanks, but all tanks within an experiment had similar patterns (Fig. 2). According to AIC, increased temperature resulted in increased WC chl *a* concentration; however, p -values were not significant (Table 2). There were no phytoplankton blooms associated with the nutrient treatments (AIC, Table 2).

3.4. Macrophyte response metrics

3.4.1 Green macroalgae (GMA)—Mean values ($\pm$ se) of total GMA biomass ranged between 34 ± 5 and 45 ± 8 g dw (Fig 3a). The response of GMA biomass to NO₃ load exhibited a significant T x N interaction ($p = 0.03$; Table 2; Fig. 3a). Visual examination of interaction plots revealed the data were highly variable with no discernable patterns; further, post-hoc analysis also did not detect significant treatment effects. Mean GMA growth rates ranged between 0.7 ± 0.1 and 1.1 ± 0.3 g dw d⁻¹ (Fig 3b). Growth rates increased as nutrient load increased (post hoc $p > 0.05$), and no significant temperature effect was observed (Table

2). Mean thallus nitrogen content ranged between 2.5 and 4.2 %N with higher values in enriched tanks. Thallus C:N (12 ± 0.7) at ambient nitrogen loading was significantly higher than at all other nitrogen loading rates, and there was no significant temperature effect (Table 2; Fig. 3c).

In the warm tanks in all three experiments, the GMA accumulations were buoyant and floated at the surface of the water, shading the plants below regardless of nitrogen load. For example, on 8/2/2013 mean $\pm$ se underwater PAR was $\sim 30\%$ lower ($63 \pm 9 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) in the warm tanks than in the cold tanks ($88 \pm 5 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$). In contrast, in the cold water tanks (regardless of nitrogen load), algae remained intercalated between seagrass shoots and did not directly shade the seagrass. This was also observed in a separate preliminary experiment that was conducted in the dark to eliminate photosynthetically-derived O_2 production (data not shown).

3.4.2. *Zostera japonica*—A variety of metrics were evaluated to examine the response of *Z. japonica* to temperature and nitrate loading (Table 2, Fig. 4). AIC indicated that only temperature influenced 4 of the 5 metrics examined (though in most cases p-values were > 0.05 , Table 2). Overall, leaf biomass for all experiments ranged between 7 and 26 mg dw sht^{-1} ; and mean leaf biomass in the warm treatment was about 30% lower than in the cold treatment (Fig 4). Likewise, new leaf C:N for all experiments ranged between 8.8 and 11.9; but mean new leaf C:N for the cold treatments was significantly larger than the warm treatment (Table 2, Fig. 4). New leaf nitrogen content ranged between 3.2 and 4.6 %N; while measured leaf growth rates ranged between 0.1 and 0.2 mg dw $\text{sht}^{-1} \text{ d}^{-1}$. Nitrate load had no effect on any of the metrics except old leaf C:N according to AIC; although p-values were not significant (Table 2).

3.4.3. *Zostera marina*—The AIC indicated that temperature was the variable influencing 5 of the 11 metrics examined, while interactions between temperature and NO_3 loading influenced the other 6 metrics (Table 2). Plants at 10 °C had larger leaf biomass than at 20 °C and plants at ambient NO_3 loading had higher biomass than under enriched treatments at 10 °C, but not at 20 °C (Fig 5A). At 20 °C, mean shoot survival was 12–15% lower than at 10 °C (Fig 5B), but there was no effect of NO_3 load. Observations revealed that all of the shoots that died exhibited decayed meristems (Supplemental Fig 2) similar to reports from Burkholder et al. (1992), although there was no relationship to nitrogen loading. Plants at 10 °C had larger maximum leaf area index than plants at 20 °C ($p = 0.0041$; Table 2). The AIC results demonstrated an effect of temperature on leaf width (Table 2), with the interaction plot showing a 2 mm difference between the 10 and 20 °C treatments (Fig 5C, Supplemental Fig. 2); however, p-value was > 0.05 . Mean leaf growth rates at 20 °C were about 2–4 mg dw $\text{sht}^{-1} \text{ d}^{-1}$ higher than at 10 °C ($p = 0.0053$; Table 2), but no differences between nutrient loading treatments were detected (Fig. 5D; Table 2).

Whole-shoot wasting disease index (mean % infection of all leaves) exhibited significant temperature by nutrient interaction (Table 2). Visual evaluation of the interaction plots (Fig 6A) found low wasting disease index (1–2%) at 10 °C and at ambient and low NO_3 loading. However, at 10 °C and medium or high NO_3 loads, the wasting disease index was about 10%, comparable to that in the 20 °C treatments (Figure 6A). The mass of new leaf tissue

per unit area (mg dw cm^{-2}) is used in the Nutrient Pollution Index (NPI) calculation and exhibited a significant T x N interaction (Table 2). Visual evaluation of the interaction plot indicates that in the 20 °C treatments leaf mass was fairly constant at around 2 mg dw cm^{-2} regardless of NO_3 load (Fig 6B). In contrast, *Z. marina* tissues at 10 °C exhibit a decrease from $2.6 \text{ mg dw cm}^{-2}$ at ambient NO_3 to about $2.2 \text{ mg dw cm}^{-2}$ at high NO_3 loads (Fig 6B). New leaf tissue nitrogen content ranged between 1.4 and 3.8 %N.

3.5. Integrative metrics

The new leaf NPI data exhibited significant temperature and NO_3 effects, but the T x N interaction term was not statistically significant ($p = 0.08$; Table 2). The new leaf NPI for plants at 20 °C ranged between 1.2 and $1.3 \text{ \%N (mg dw cm}^{-2})^{-1}$ regardless of NO_3 loading rate (Fig 6C). In contrast, the 10 °C plants exhibited a strong increase from 0.8 %N ($\text{mg dw cm}^{-2})^{-1}$ at ambient loading to $1.34 \text{ \%N (mg dw cm}^{-2})^{-1}$ at high loading rates (Fig 6C).

Macrophyte community structure, as percent of total biomass (Fig. 7) exhibited shifts in species dominance. At 10 °C, increased nitrate loading did not influence the biomass or percent contribution to the total biomass (Fig. 7A through D). In contrast at 20 °C and ambient NO_3 loading, GMA accounted for 60 % of the total biomass (Fig 7E), even though the actual biomass was about the same as in the cold treatments. Average GMA biomass increased by about 6 to 10 g dw in the 20 °C and low and medium NO_3 loading treatments, accounting for almost 70% of total biomass in these treatments (Figs. 7F and 7G). The shift in community dominance was primarily a result of reduced seagrass biomass, but GMA biomass also increased in the warm low and medium treatments (Figs. 7F and 7G). *Zostera marina* biomass decreased by about 5 to 10 gdw, but it still accounted for 25 to 30% of total macrophyte biomass (Fig. 7E through 7H). *Zostera japonica* biomass at 20 °C for the medium and high NO_3 loading rates was reduced, accounting 4 to 6.5 % of total macrophyte biomass (Fig. 7G and H).

Gross ecosystem production rates at 10 °C were 20 to 50% higher than at 20 °C (Table 2 & 3) regardless of NO_3 loading rate. At 20 °C there appears to be an increase in GPP with enrichment but it was not statistically significant (Table 2). According to AIC criteria, R_d rates were affected by temperature but not NO_3 loading treatments (Table 2 and 3). Since animals were purposefully excluded from the mesocosms, it was not surprising that tanks were highly autotrophic ($P \gg R$; Table 3); however at least one treatment combination had a P:R of 1.6, implying that respiration may have been more important under those conditions (Table 3). The strong temperature effect observed in the production and R_d data also carried over into the P:R data, such that at 20 °C, tanks had lower P:R values.

4. Discussion

Seagrass communities have long been considered susceptible to cultural or anthropogenic eutrophication and are negatively impacted by processes and events that result in decreased water clarity leading to light limitation for benthic macrophyte communities. Increased nutrient loading is frequently attributed as a driver for the development of eutrophication responses (Nixon 1995; Andersen et al. 2006; Nixon 2009), but temperature clearly has a large influence on the degree of system degradation. Expression of eutrophication indicators

such as community shifts, loss of seagrass, and formation of floating algal mats were related to warm temperatures and generally not to nutrient loading. There is a suite of factors, most notably residence time (Valiela et al. 1997; Valiela et al. 2000; Nixon et al. 2001; Bricker et al. 2008) or OM export (Barrón et al. 2003), that can ameliorate some symptoms of eutrophication. Our expectation was that increased NO_3 loading would stimulate algal production and the resultant algal dominance would cause seagrass condition and biomass to decline within the mesocosms. Although changes in community structure were observed the magnitude of response was smaller than expected and some eutrophication symptoms were not expressed, particularly phytoplankton blooms, organic matter build up and hypoxia. Consequently, these short residence time experiments exhibited few eutrophication symptoms, despite a large gradient of NO_3 loads to the system. Our findings support the hypothesis that residence time and thermal regime can buffer the expression of some eutrophication symptoms and requires further assessment.

4.1. Community structure

The nutrient loading hypothesis predicts shifts in macrophyte community dominance related to increased nutrient loading and increased competitive light interactions (Short et al. 1991; Bricker et al. 2008; Brauer et al. 2012). However, the response of these experimental macrophyte mesocosms highlights that temperature may also play a substantive role in the expression of eutrophic symptoms. The 10 °C and ambient NO_3 loading treatment were chosen as representative of conditions in temperate, upwelling influenced PNW open-coast estuaries. Despite a 6-fold increase in NO_3 loading at the cold temperature (10 °C) there was no alteration of macrophyte community dominance. In contrast, at 20 °C and ambient NO_3 loading, a 40% decrease in biomass of both seagrass species relative to the 10 °C treatments resulted in GMA dominance increasing by about 10% (Fig. 7E). Furthermore, as NO_3 loading increased at 20 °C GMA biomass exhibited a convex pattern with lower biomass at ambient and high nutrient loads and higher biomass at low and medium nutrient loads. Despite the convex pattern, GMA biomass was not all that different between treatments varying by about 10 gdw (Fig. 7E through 7H). Thus, loss of seagrass possibly from shading (see below) and not increases in GMA biomass accounted for changes in dominance. The relatively weak response of GMA biomass to nutrient loading was unexpected and we theorize that the community response was more a function of how that biomass was distributed in the water column (floating vs intercalated).

Epiphytic algal communities respond strongly to water column nutrients and have been demonstrated to impact seagrasses including *Z. marina* (see review by Nelson 2017). Although, epiphyte metrics have been commonly used as indicators of nutrient loading (Nelson 2017), they were not directly assessed in these experiments. Epiphytes were present on the *Z. marina* blades during these experiments but were not directly quantified. We quantified the MAF on the walls of the tanks and assumed that the epiphyte community was similar in magnitude. However, we could not include the MAF in assessment of community alterations because the units were not comparable ($\text{mg chl } a \text{ m}^{-2}$ vs gdw m^{-2}); which is a common issue in the literature (Nelson 2017). Although epiphytes were present in the mesocosms, it is unlikely that they played a strong role in altering community structure. MAF standing stock, a surrogate for epiphytes, were 2 fold higher at 10 °C than at 20 °C

which is not consistent with the community shifts, which were only observed at 20 °C (Fig. 7).

Decreases in *Z. marina* biomass were small as a result of shoot mortality, and this species still accounted for 25 to 30% of total biomass. None of the 240 *Z. marina* plants exposed to 10 °C died during these experiments regardless of NO₃ loading rate. In contrast, at 20 °C there was notable mortality of *Z. marina* shoots that was not related to NO₃ loading, since plants at ambient loading also died. Work from other regions found that high nitrogen levels can be toxic to *Z. marina* (Burkholder et al. 1992; van Katwijk et al. 1997; Brun et al. 2002; Invers et al. 2004). However, the lack of a direct NO₃ toxicity effect in this experiment is consistent with previous work where plants exposed to 1000 µM NO₃ or NH₄ exhibited no detectable stress or mortality (Kaldy 2012). Likewise, Hessing-Lewis et al. (2015) exposed eelgrass plants to very high nutrient loads and found little evidence for nutrient effects. Some seagrass populations may be more susceptible to thermal or other stressors than to direct nutrient stressors.

Losses of *Z. japonica* were more substantial, and the species accounted for only 4 to 8% of the total biomass. In this experiment, *Z. japonica* shoot-specific growth rates were similar to previously published rates at 8 °C, but were 3 fold lower than growth rates measured at 20 °C (Kaldy et al. 2015), indicating that some other factor was limiting growth. We hypothesize that light limitation caused by floating macroalgae resulted in the reduced *Z. japonica* biomass. Floating green algal mats have often been reported to shade and smother seagrass under eutrophic conditions (Harlin, 1995; Burkholder et al. 2007). During these experiment we routinely observed floating green algal mats over-topping the seagrass plants, but only under the 20 °C treatment, irrespective of NO₃ load. Because *Z. japonica* is diminutive relative to *Z. marina*, it is more susceptible to light limitation by such floating mats. We hypothesize that as cold water was added to the tank and warmed, changes in solubility caused outgassed O₂ to accumulate under the algae, ultimately leading to floating mats. Thus water motion and exchange may also influence the development and persistence of floating algal mats.

Wasting disease has been implicated in large scale losses of *Z. marina* in North America and Europe (Rasmussen 1977), and warm water temperatures favor the occurrence of the pathogen *Labyrinthula zosterae* (Short et al. 1987; Muehlstein et al. 1991). Generally, wasting disease has not been very prevalent within Yaquina Bay (<1%; Kaldy unpublished data), although it has been hypothesized as a factor contributing to *Z. marina* losses in Morro Bay, CA. (R. Thom, pers. comm.) and Westcott Bay, WA. (Schanz et al. 2010). Kaldy (2014) concluded that wasting disease was correlated with warm thermal regimes (e.g. >20 °C), but found no apparent influence from nutrient loading. The Wasting Disease Index (WDI) data collected from the present experiments also supports a temperature effect, but this was confounded by a significant T x N interaction term. We found that warm temperatures increased WDI, but under moderate to high nitrogen loads (20–50 mmols d⁻¹; Table 1), even plants at 10 °C can have increased WDI. Eelgrass communities subjected to increased thermal or nutrient stressors from either climate change or land-use management alterations may be more susceptible to wasting events.

Based on empirical field studies, Lee et al. (2004) suggested that the ratio of leaf nitrogen to leaf mass (NPI) of mature tissue could be used as an indicator of nitrogen availability. However, application of the NPI may be somewhat limited by site-specific environmental conditions. In a field verification study, the NPI failed to detect the north-south nutrient loading gradient in Barnegat Bay-Little Egg Harbor, NJ during summer months (Kennish and Fertig 2012), a period with water temperatures in the range of 19 – 24 °C (Kennish et al. 2009). In comparison, our data on new leaf NPI exhibited a strong linear relationship with nitrate loading rates at 10 °C; while at 20 °C there was no relationship between new leaf NPI and NO₃ loading (Fig. 6c). Water temperature appears to play a role in the expression of a nutrient effect and may be an important factor to consider in the application of the NPI. The NPI data presented here was for the youngest emergent leaf (leaf tip was above the bundle sheath), which may not have reached full cellular expansion (growth), especially during rapid leaf elongation at 20 °C. However, new leaf tissue weight was generally less variable than the areal mass of mature leaf tissue from the same plants (Supplemental Figure 3). Newly formed tissue was targeted because, as the site of active cell division, it is most likely to reflect the plant's response to the experimental conditions imposed in the mesocosms. The significant T x N interaction effect for new leaf weight area (mg dw cm⁻²), the denominator in the NPI calculation (%N (mg dw cm⁻²)⁻¹), shows that the plant response for this variable was dependent on both factors. NPI calculated with mature leaf areal weights did not exhibit the expected relationship with increased load (Supplemental Figure 4) and the relationships were not statistically robust. We are encouraged that the new leaf NPI has potential as a metric of eutrophication, but additional studies are needed to determine if it is applicable across a range of thermal regimes.

One caveat of the present work is that, due to the short treatment duration (3 weeks), it is unlikely that the experimental communities had reached equilibrium with respect to community response. For example, given a longer experiment, morphologically plastic leaf characteristics may have completed a dramatic shift toward narrow leaves, which could be a diagnostic expression of thermal stress. Frequently, alteration of leaf morphology is considered a response to light limitation (Lee et al. 2007; Ralph et al. 2007), although temperature response has been reported for some species (York et al. 2013). Unfortunately, the experiment was not long enough to allow complete turn-over of standing leaves. It is noteworthy that the short-term trajectory of response is consistent with longer term experiments (Short et al. 1995; Taylor et al. 1995), and that the communities appear to be more sensitive to thermal stress than to nutrient loading per se. Longer-term mesocosm experiments incorporating both thermal and nutrient stress as well as more complex ecosystem components (e.g. consumers) could provide better insight on how rapidly flushed systems might respond to warming temperatures and increased nutrients.

4.2. Ecosystem metabolism

We evaluated ecosystem metabolism as a proxy for eutrophication expecting that a eutrophic system would be dominated by high rates of production and high respiratory demand from remineralization of accumulated organic matter. Our ecosystem metabolism data demonstrated that all of the treatments were highly net autotrophic with P:R > 1. There appears to be an increase in production with nutrient loading at 20 °C (Table 3), but

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differences were not statistically significant. Low statistical power may have limited our ability to detect weak eutrophication patterns within the data. A weak eutrophication response is corroborated by the high DIN:ON and the low DOC concentrations ($< 0.2 \text{ mmol l}^{-1}$), showing that much of the added N and resultant production (DOC) was flushed from the system. Although, plant tissue %N confirmed that some of the added N was incorporated into biomass, these additions did not appear to stimulate blooms of macro or microalgae (phytoplankton or MAF), perhaps as a result of strong P limitation. In contrast, other mesocosm work found that ecosystem production and respiration were increased by nutrient additions, and that accumulated sediment OM contributed to ecosystem respiration (Spivak et al. 2009). Unfortunately, the role of water residence time in the accumulation of OM was not reported. Although we did not see a strong nutrient response, our data consistently exhibit a strong thermal response. Based on AIC, ecosystem metabolism was strongly influenced by increased temperature. Surprisingly production decreased at 20°C relative to 10°C (Table 3); we hypothesize that phosphorus or some other resource may have been limiting. This is surprising since both P and R_d are known to increase with warming (Lee et al. 2007), and there was increased variability in the ecosystem R at 20°C (Table 3). We expected to document strong patterns of eutrophication in these mesocosm systems. However, the patterns that emerged were subtler than anticipated given the nutrient loads highlighting the need for sensitive indicators of eutrophication that perform across a wide range of environmental drivers. Ecosystem metabolism may be a better metric for detecting weak patterns in systems that are near the residence time threshold for eutrophication expression than for systems with high turn-over.

4.3. Residence Time

These mesocosm conditions were designed to contrast with previous experiments (Burkholder et al. 1992; Short et al. 1995; Taylor et al. 1995; Taylor et al. 1999) that were conducted to mimic longer residence time systems. Low exchange rates of about ~5–20 % result in long residence times and warm water temperatures ($> 20^\circ\text{C}$) that favor development of eutrophic conditions (Valiela et al. 1997; Valiela et al. 2000; Nixon et al. 2001; Bricker et al. 2008). The short residence time (~0.5 d) and large simulated tides used in the current experiments are consistent with conditions for open coast estuaries in upwelling regions (Lee and Brown 2009), which tend not to exhibit strong eutrophication symptoms such as seagrass loss, hypoxia and fish kills, despite large nutrient loads (Hessing-Lewis et al. 2015; Bricker et al. 2007, 2008). Manipulative field experiments have also found little evidence for nutrient and macroalgal effects on eelgrass, and researchers concluded that water motion and tidal submersion alleviated eutrophication impacts (Hessing-Lewis et al. 2015). Likewise, phytoplankton blooms observed within the estuary are also related to upwelling/downwelling events (Brown and Ozretich 2009). Our results show that despite the favorable light, temperature and massive nutrient loading conditions, the eutrophication response was subtle or not detectable with our experimental design. Clearly, further work is required to understand the role of temperature in the development of strong eutrophication responses to nutrient loading. Another obvious research need is the empirical determination of threshold residence time conditions that lead to eutrophication response for given temperature and nutrient regimes, and the scaling of such empirical work to estuarine systems.

4.4. Summary and Conclusions

Experimental estuarine macrophyte communities subjected to increased nutrient loading generally exhibited little evidence of eutrophication response, although at 20 °C there were community shifts consistent with the nutrient loading hypothesis. However, there was no evidence of other eutrophication symptoms (e.g. hypoxia, DOC build up, plankton blooms, etc.) presumably as a result of the short system residence time. In general, thermal regime appeared to have a more robust influence on most response metrics than did nutrient load. Teasing apart the influence of temperature and nutrients on the expression of eutrophication symptoms will require complex multi-stressor experiments and use of indicators that are sensitive across a broad range of conditions. Systems currently subjected to cool temperatures, high nutrient loads, and short residence time may be resilient to expressing many symptoms of eutrophication, or the expression may be through weak linkages that are difficult to detect. Consequently, altered thermal regimes associated with climate change may dramatically influence system expression of eutrophication responses in estuarine macrophyte communities of temperate upwelling influenced systems such as the Pacific Northwest.

These experiments focused on the role of increased nitrogen loading to the systems, but do not address the influence of phosphorus (P) loading or limitation. Although estuarine systems are generally considered N limited, the artificial loadings used here may have pushed the systems to P limitation, resulting in limited community response. There is a need to evaluate turn-over time thresholds for ambient loading and nutrient enrichment that lead to development of eutrophication symptoms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Experiment: Jun 10 - Jul 1

Jul 5 - Aug 9

Sep 9 - 30

Nutrient treatments

Mesocosm design

Figure 1.

Schematic diagram of experimental design including replicate mesocosm units. Temperature and nutrient load combinations are represented by colored boxes. Each combination represents a single mesocosm unit. See text for additional details. Dashed lines indicate labels for various features. Solid black lines with arrows indicate magnitude and direction of water flow within the system. Wide gray arrows indicate water motion via pumps; internal circulation and heating/cooling.

Figure 2.

Time series of water column chl *a* ($\mu\text{g L}^{-1}$) sampled daily during the experiments. Each panel (A– C) represents an individual experiment. Data gaps were the result of not sampling.

Figure 3. Green macroalgae response metrics. (A) Interaction plot of GMA Biomass (g dw; mean $\pm$ se; $n = 3$). (B) GMA growth (g dw d⁻¹) response to nutrient load (mean $\pm$ se; $n = 6$). (C) GMA thallus C:N response to nutrient load (mean $\pm$ se; $n = 6$).

Figure 4. *Zostera japonica* metrics response to temperature, n = 12. (A) Mean ($\pm$ se) leaf biomass (mg dw sht⁻¹) relative to the temperature treatments (B) Mean ($\pm$ se) new leaf tissue C:N relative to temperature.

Figure 5. *Zostera marina*

metrics response to temperature –nutrient treatments (n = 3). (A) Interaction plot for mean ($\pm$ se) leaf biomass (g dw sht⁻¹) relative to temperature and nutrient treatments. (B) Interaction plot for shoot survival ($\pm$ se) as a percentage of initial relative to temperature and nutrient conditions. Note symbols at medium loading rates directly overlap. (C) Interaction plot for mean ($\pm$ se) leaf width (mm) for the temperature and nutrient combinations. (D) Interaction plot for mean ($\pm$ se) leaf growth (mg dw sht⁻¹ d⁻¹) for the temperature and nutrient combinations.

Figure 6. *Zostera marina*

physiological metrics, $n = 3$. (A) Interaction plot for mean ($\pm$ se) whole shoot wasting disease index for temperature and NO_3 treatments. (B) Interaction plot for mean ($\pm$ se) leaf weight per area (mg dw cm^{-2}) for temperature and NO_3 treatments. (C) Interaction plot for mean ($\pm$ se) Nutrient Pollution Index ($\% \text{N (mg dw cm}^{-2})^{-1}$) in relation to temperature and NO_3 treatments.

Figure 7. Macrophyte community structure expressed as a percentage (pie diagrams) of total macrophyte biomass and mean ($\pm$ se) biomass (gdw) in upper right corner of each panel ($n = 3$). Values were averaged over triplicate experimental runs. Microalgae were not included because standing stock chl *a* units were not comparable. Each panel (A through H) represents a single temperature (cold = 10 °C or warm = 20 °C) and NO₃ load treatment (Ambient, Low, Medium, and High). GMA = green macroalgae, Zm = *Zostera marina* and Zj = *Z. japonica*.

Table 1

Summary of daily (n=21) average ($\pm$ SD) nutrient concentrations (μ M) and estimated loading rates (mmols d^{-1}) and ratios to the mesocosms during the three experiments. Daily NH_4 concentrations were consistently $< 3\mu\text{M}$ while PO_4 were consistently $< 1\mu\text{M}$ in all experiments.

		Units	Inflow	Ambient	Low	Medium	High
Expt 1	NO_3	μM	NA	7 ± 10	10 ± 11	23 ± 10	58 ± 14
	NO_3	mmols d^{-1}	NA	5 ± 7	7 ± 8	17 ± 7	42 ± 10
	DIN:P	Unitless	NA	15 ± 8	30 ± 6	112 ± 54	330 ± 200
	ON	μM	NA	17 ± 7	16 ± 5	17 ± 5	22 ± 6
	DIN:ON	Unitless	NA	0.6 ± 0.6	0.8 ± 0.7	1.7 ± 1.0	3.2 ± 1.4
Expt 2	NO_3	μM	NA	16 ± 7	19 ± 7	33 ± 9	68 ± 18
	NO_3	mmols d^{-1}	NA	11 ± 5	14 ± 5	24 ± 7	49 ± 13
	DIN:P	Unitless	NA	24 ± 4	31 ± 4	54 ± 14	120 ± 57
	ON	μM	NA	16 ± 3	17 ± 3	22 ± 9	25 ± 11
	DIN:ON	Unitless	NA	1.2 ± 0.7	1.2 ± 0.5	1.8 ± 1.0	3.3 ± 2
Expt 3	NO_3	μM	12 ± 4	7 ± 3	11 ± 4	27 ± 7	61 ± 16
	NO_3	mmols d^{-1}	8 ± 3	5 ± 2	8 ± 3	19 ± 5	44 ± 12
	DIN:P	Unitless	26 ± 1	18 ± 3	32 ± 4	74 ± 19	180 ± 55
	ON	μM	NA	13 ± 2	13 ± 3	16 ± 3	21 ± 6
	DIN:ON	Unitless	NA	0.6 ± 0.2	0.9 ± 0.3	1.9 ± 0.4	3.4 ± 1.6

Table 2

Summary of statistical analysis using AIC to evaluate the models. AIC criteria were used to identify factors that affected response variables (statistical significance); p-values from specific models for significant factors are provided.

Plant Type	Variable	Best Fit Model			Significant factors (p value)	Sample size (n)	units
		nutrient	temperature	additive			
MAF ¹ Phyto ² GMA ³	chl <i>a</i>		X		T (0.0097)	120	mg m ⁻²
	chl <i>a</i>		X		>0.05	400	µg l ⁻¹
	Biomass			X	TxN (0.03)	24	g dw tank ⁻¹
<i>Z. japonica</i>	Growth	X			N (0.02)	24	g dw d ⁻¹
	C:N	X			N (<0.0000)	24	unitless
	Leaf biomass		X		T (0.0066)	24	mg dw shr ⁻¹
	Growth		X		>0.05	24	mg dw shr ⁻¹ d ⁻¹
	New leaf C:N		X		T (0.019)	24	unitless
<i>Z. marina</i>	Old leaf C:N	X			>0.05	24	unitless
	RR C:N		X		>0.05	24	unitless
	Leaf biomass			X	T (<0.0000); N (0.047)	24	gdw shr ⁻¹
	Growth		X		T (0.0053)	115	gdw shr ⁻¹ d ⁻¹
	Leaf width		X		>0.05	120	mm
Wasting Disease Index	Survival		X		T (0.0195)	24	shoots
	Leaf Area Index		X		T (0.0041)	120	cm ² m ⁻²
	New leaf weight area			X	T (0.006)	120	% whole shoot infected
				X	TxN (0.02)		
				X	T (0.0001)	115	mg dw cm ⁻²
New leaf NPI				X	N (0.006)		
				X	TxN (0.03)		
				X	T (0.014)	115	% N (mg dw cm ⁻²) ⁻¹
New leaf C:N				X	N (0.007)		
				X	T (0.04)	118	unitless
				X	N (0.0011)		

Plant Type	Variable	Best Fit Model			Significant factors (p value)	Sample size (n)	units
		nutrient	temperature	additive			
Metabolism	Old leaf C:N			X	T (0.011) N (0.0059)	117	unitless
	RR C:N		X		T (<0.0000)	118	unitless
	P		X		T (<0.000)	23	mg O ₂ l ⁻¹ h ⁻¹
	R		X		>0.05	23	mg O ₂ l ⁻¹ h ⁻¹
	P:R			X	T (0.0006)	23	unitless

¹MAF - microalgal flora
²Phyto - phytoplankton
³GMA - green macroalgae

Table 3

Mean ($\pm$ se) ecosystem metabolism rates normalized to volume of experimental mesocosm tanks (n=3). Mean ($\pm$ se) tank volume was 350 ± 5 liter. Values were measured at the end of each experiment and represent metabolism of all ecosystem components (e.g. seagrass, algae, phytoplankton, MAF, etc.).

Temperature	Nutrient Loading	Production mg O ₂ tank ⁻¹ h ⁻¹	Respiration mg O ₂ tank ⁻¹ h ⁻¹	P:R
10 °C	Ambient	241 (1)	51 (2)	4.76 (0.2)
	Low	264 (11)	58 (1)	4.58 (0.1)
	Medium	254 (7)	52 (2)	4.91 (0.1)
	High	249 (15)	50 (8)	5.21 (0.7)
20 °C	Ambient	95 (25)	67 (25)	1.59 (0.3)
	Low	153 (24)	73 (23)	2.95 (1.4)
	Medium	204 (30)	50 (14)	4.33 (0.6)
	High	164 (35)	56 (4)	3.01 (0.8)