



Epiphyte load and seagrass performance are decoupled in an estuary with low eutrophication risk

Jennifer L. Ruesink

Department of Biology, University of Washington, Box 351800, Seattle, WA, 98195-1800, USA

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ABSTRACT

Seagrass can decline due to blooms of algae following either loss of top-down control or increase in bottom-up nutrient delivery. Macroalgae negatively affect seagrass especially at high ratios of algal biomass to seagrass size. In contrast, for microalgae, epiphyte loads that affect seagrass performance are not well synthesized. Competition has been demonstrated primarily following nutrient or food web manipulations, which increase epiphyte load from background levels, rather than through experimental removal of epiphytes. Although the Willapa Bay (Washington, USA) is an estuary with low eutrophication risk, microalgae reach large biomass in summer, up to 60% of the mass of their intertidal seagrass host (eelgrass, *Zostera marina*). Based on a novel field technique, experimental reduction of epiphytes did not accelerate growth ($\text{g g}^{-1} \text{d}^{-1}$) of *Z. marina*. Neither did two other manipulations that were expected to improve resource access: removing nearby shoots or older, epiphytized leaves. Instead, removing older leaves slowed growth from $2.3\% \text{d}^{-1}$ to $1.8\% \text{d}^{-1}$, and removing lightly-epiphytized younger leaves slowed growth to $1.7\% \text{d}^{-1}$. To evaluate top-down and bottom-up causes of epiphyte load, two widespread methods were used. Observationally over time, epiphyte load was positively correlated with eelgrass leaf emergence rate and unrelated to mesograzers; however, a crossed experiment reducing crustacean mesograzers and adding nutrients indicated top-down control, since chlorophyll-a in epiphytes increased with grazer reduction and no added nutrients (deterrent \times nutrient interaction). At current epiphyte loads in the Willapa Bay, intertidal *Z. marina* tolerates seasonally-abundant epiphytes coinciding with rapid leaf emergence, thus producing fresh leaves where microalgal competition cannot be detected.

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1. Introduction

Both micro- and macro-algae can form blooms that competitively exclude seagrasses (Duarte, 1995), but this straightforward ecological interaction has been complicated by evidence that blooms result from multiple factors and exert context-dependent effects on seagrasses. Consequently, both the causes and consequences of algal blooms in seagrass beds require case-specific evaluation. The causes of algal blooms have been variously attributed to both top-down and bottom-up controls. With top-down forcing, a trophic cascade from overfishing of top predators allows mesopredators to become abundant and to limit mesograzers (gastropods and crustaceans) that would keep algae in check, and thus overfishing links to both algal blooms and seagrass declines (Heck et al., 2000; Hughes et al., 2013). With bottom-up forcing, competitors gain an advantage over seagrass because of release from nutrient limitation in the water column (Duarte, 1995; Hughes et al., 2004). The relative contribution of bottom-up and top-down pathways in particular seagrass systems has been tested in several cases involving both slow-release nutrient addition and reduction of mesograzers, with

grazing of epiphytes a critical component (Heck et al., 2000; Verhoeven et al., 2012; Whalen et al., 2013; Reynolds et al., 2014; Duffy et al., 2015). In contrast to this experimental evidence, **epiphyte distribution and abundance can change spatiotemporally in concordance with factors other than grazers, including seasonal changes in light or temperature, eutrophication, and variation in seagrass leaf turnover (Bulthuis and Woelkerling, 1983; Borum, 1987; Nelson and Waaland, 1997).** In fact, in observational studies over seasons, epiphytes and grazers can be positively correlated due to similar seasonal dynamics (Nelson and Waaland, 1997; Fong et al., 2000), and mesograzers may release epiphytes from light or nutrient-limitation (Jaschinski and Sommer, 2010).

Competition experiments between macroalgae and seagrass were synthesized recently via meta-analysis (Thomsen et al., 2012), without reference to microalgal epiphytes. Impacts scaled with macroalgal density and were inversely related to seagrass size, but were overall rather weak (Thomsen et al., 2012). In some cases, macroalgae may even benefit seagrass by harboring mesograzers that clean seagrass leaves (Whalen et al., 2013). Epiphytes growing on seagrass leaves have long been implicated to restrict seagrass access to light and carbon, with negative effects more obvious when resources are below saturating levels (Sand-Jensen, 1977). Unlike macroalgae, however, microalgae have not been directly manipulated in seagrass field experiments to compare

E-mail address: ruesink@u.washington.edu.

responses with and without competitors. Rather, manipulations of grazers or nutrients tend to increase epiphytes relative to background levels (Hughes et al., 2004; Duffy et al., 2015).

In this study, the causes and consequences of epiphyte loading on intertidal eelgrass (*Zostera marina*) were examined in a context of robust eelgrass populations and low eutrophication risk. The study paired two approaches that are widespread in seagrass-epiphyte studies: 1) an observational study of environmental correlates of epiphytes, and 2) a crossed experimental manipulation of nutrients and grazers. The observational approach cannot be used alone to derive mechanistic conclusions but does define natural variability in epiphyte load. If bottom-up factors control seasonal variability, then positive correlations are expected between epiphytes, light, and nutrients. Alternatively, epiphyte load would be negatively correlated with leaf turnover under a scenario of host control. Top-down control could result in an inverse relationship between epiphytes and grazers. The companion experiment, involving mesograzers and addition of slow-release nutrients in the field, examined two mechanisms that could limit epiphyte load in Willapa Bay during its summer season of peak biomass. In addition to these methods, a novel direct removal of microalgae in the field was employed to determine the consequences of standing levels of epiphytes. Effects of epiphyte load were tested in comparison to other manipulations selected to explore additional mechanisms that could modify resources available for seagrass growth. Specifically, shoots were thinned and outer, epiphytized leaves removed in treatments that were expected to increase resource availability, and young, relatively clean leaves were removed to evaluate their contribution to whole-shoot performance.

2. Material and methods

2.1. Study site

The study took place in an eelgrass (*Z. marina*) bed near the center of its estuarine distribution in Willapa Bay (Washington, USA; N46.475°, W124.022°). *Z. marina* occupies 10% of the area of the bay (>3000 of 34,904 ha; Borde et al., 2003; Ruesink et al., 2006), primarily between +0.5 and −1 m relative to mean lower low water (MLLW; Ruesink et al., 2010). The bay receives tidally-advected nutrients from upwelled ocean water but has low human population density in the watershed and rapid flushing, therefore a “low overall eutrophic conditions rating” (Bricker et al., 2007 p. A153). Within *Z. marina* tissue, nitrogen below 1.8% indicates nutrient limitation (Duarte, 1990). In contrast, tissue nitrogen at the study site greatly exceeds this level (>3%, Duffy et al., 2015), although varying spatially in Willapa Bay from 1.5% to 3.2% in summer (Ruesink et al., 2015). Consistent with this assessment of available nutrients, *Z. marina* growth did not respond to sediment nutrient addition (Wagner et al., 2012). The health of eelgrass populations in the bay is also suggested by resilience to pulse disturbances (Wisehart et al., 2007; Ruesink et al., 2012), including shellfish aquaculture that can overlap with eelgrass but was not present within the target eelgrass bed.

2.2. Seasonal surveys of epiphytes and mesograzers

Epiphytes and mesograzers were surveyed at one- to three-month intervals for a total of 10 times between June 2011 and September 2012 at an elevation near MLLW. Epiphytes primarily consist of microalgae at this tidal elevation and polyhaline conditions (salinity of 18–29 depending on river flow). Macroalgae (*Enteromorpha* spp., *Polysiphonia* spp.) are generally not attached to eelgrass blades and overall are uncommon in Willapa Bay relative to other US west coast estuaries (Hessing-Lewis and Hacker, 2013). At each sampling time, 4–5 *Z. marina* shoots were collected haphazardly along 50 m to evaluate epiphyte load. Epiphytes were collected from leaves extending above the outer sheath by pulling a glass slide along both sides of each leaf and drying the slurry from each leaf separately (60 °C for 1 week). Epiphytes were weighed, along with the corresponding dried leaves and sheath, and epiphyte dry mass standardized by shoot dry mass. To account for variation in number of leaves among shoots across seasons, this metric of epiphyte load was based on the five most recent leaves for each shoot, including the leaf sheaths.

To quantify the mesograzers assemblage, 4–5 eelgrass samples were collected during each sampling time, with each sample consisting of the shoots caught within a mesh bag lowered over the shoots in shallow water (bag diameter of 20 cm, 80 µm mesh; Reynolds et al., 2014). Eelgrass material was agitated and gently scraped in freshwater to remove mesograzers, then dried and weighed to provide a means of standardizing mesograzers by the amount of host material. The water was sieved (0.5 mm), and the trapped mesograzers were identified and counted by size class, using a series of nested sieves to estimate epifaunal biomass (Edgar, 1990; Reynolds et al., 2014).

Seasonal patterns of *Z. marina* growth throughout Willapa Bay were determined from intensive sampling in 2004 (Ruesink et al., 2010). For each sample at each of seven sites, 30–50 shoots were marked with holes near the top of the leaf sheath, and 2–5 days later the shoots were examined for new, unmarked leaves extending beyond the original mark. The fraction of plants with newly-emerged leaves, standardized by the time between marking and collection, represents leaf emergence rate and is also the inverse of the leaf plastochron interval (Herbert and Fourqurean 2009).

Epiphyte load was examined with respect to five total variables. Two of these predictors were abiotic: daylength, and the average water temperature recorded by a nearby logging sensor (iButton, Dallas Semiconductor) on each day when the eelgrass bed was sampled. Two predictors represented the substratum availability (shoot dry mass) and turnover (leaf emergence rate) of *Z. marina*. Leaf emergence rate was interpolated from a sine wave fit to 2004 data (New leaves $d^{-1} = 0.057 \cdot (\sin(2\pi \cdot (\text{day of year} - 53)/365)) + 0.098$, $n = 39$). Finally, crustacean mesograzers density was included as a predictor for top-down control, either as the total assemblage or as one of three major taxa (isopods, caprellid amphipods, or gammarid amphipods); thus, mesograzers density was only included as a single predictor variable in any particular model but required four distinct sets of models to accommodate the four ways of defining this value. The best predictors of epiphyte load were identified through a model selection

Table 1
Importance of predictors of seasonal epiphyte load in Willapa Bay, Washington. Parameter importance is the sum of Akaike's weights across containing models in the 95% confidence set. In parentheses is shown the number of models in the 95% confidence set that contain the parameter. Mesograzers were incorporated in four different ways.

Mesograzers parameter as:	Predictors				
	Leaf emergence rate	Daylength	Mesograzers	Shoot dry mass	Water temperature
Total density	0.77 (3)	0.18 (1)	0.04 (1)	0.06 (1)	0.01 (1)
Caprellid amphipod density	0.71 (3)	0.22 (2)	0.13 (4)	0.06 (1)	0.02 (1)
Gammarid amphipod density	0.77 (3)	0.19 (2)	0.05 (2)	0.06 (1)	
<i>Idotea</i> isopod density	0.75 (3)	0.17 (1)	0.07 (2)	0.06 (1)	

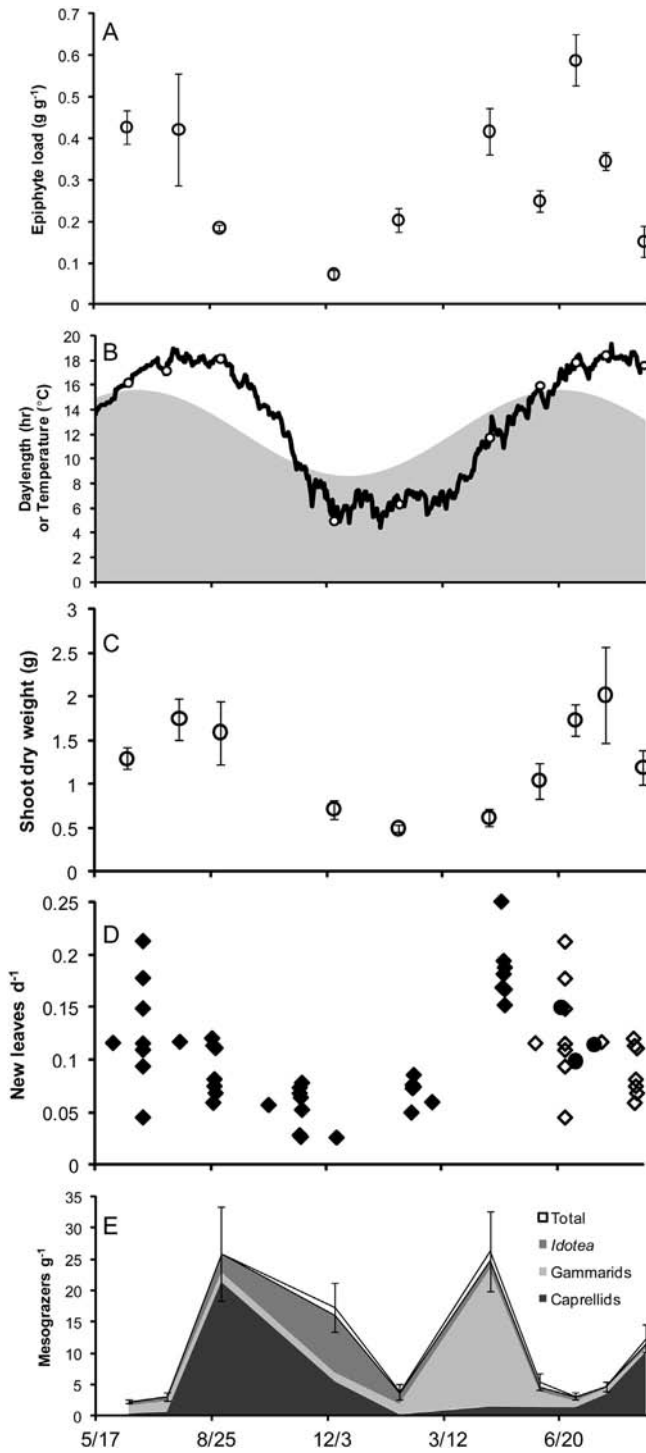


Fig. 1. Epiphytes on eelgrass (*Zostera marina*) and potential environmental predictors across 16 months in Willapa Bay, Washington. A) Epiphyte load on 5 leaves, standardized per eelgrass dry mass, B) daylength (shaded) and daily water temperature (line), C) Dry above-ground biomass per shoot, not including epiphytes, D) Leaf emergence rates, and E) Mesograzers density, standardized per eelgrass dry mass, including representation across three dominant taxonomic groups. Points in panel A, C, and E are means \pm SE ($n = 4-5$) in 2011–2012, and in D are from seven sites around Willapa Bay in 2004, in which open diamonds are duplicated from the year before, and filled circles are unmanipulated shoots in experiments in 2012. Statistical results are in Table 1.

approach, comparing a total of 24 models (for each mesograzer predictor) including a null model with intercept only. The models were restricted to at most two-way interactions among the five predictor

variables due to lack of underlying mechanism for higher-order interactions, and models did not include correlated predictors ($r > 0.55$). Shoot mass was correlated with temperature ($r = 0.83$) and daylength ($r = 0.64$), leaf emergence rate was correlated with temperature ($r = 0.57$) and daylength ($r = 0.90$), and temperature and daylength were correlated ($r = 0.87$), but leaf emergence rate and shoot mass were not ($r = 0.32$). Therefore, the most complex model included leaf emergence rate, shoot mass, and mesograzers density as main effects and all three two-way interactions. Daylength and temperature only appeared in less complex models alone or with mesograzers density.

The suite of linear models was compared by Akaike's Information Criterion, adjusted for small sample size (AICc), which penalizes more complex models (Burnham and Anderson, 2002). Results are presented in terms of the importance of each predictor, which uses the Akaike weights to construct a 95% confidence set of models, dropping the least likely models from the full set of 24. Parameter importance is the sum of model weights across all models in the 95% confidence set in which the parameter appears (Burnham and Anderson, 2002). Linear models were constructed in R (R Development Core Team 2013), followed by assessment of parameter importance using the Multimodel Inference package (MuMIn; Barton, 2015).

2.3. Epiphyte response to mesograzers and nutrient manipulations

A fully-factorial experimental design of crustacean deterrence and nutrient addition was applied to a total of 40 triangular plots (0.5 m on a side, $n = 10$ per treatment) arrayed 2 m apart along a contour of -0.3 m MLLW. As described in Reynolds et al. (2014), mesograzers were deterred from plots by placing in each corner a plaster block infused with a crustacean deterrent (carbaryl, e.g., Poore et al., 2009), which has an effective radius of about 0.6 m and thus highly localized effects (Reynolds et al., 2014). Nutrients were added via three mesh bags with a total of 750 g slow-release Osmocote (14–14–14) per plot. This method has been shown to increase nitrogen content in seagrass tissue when background nutrients are limiting to growth (Reynolds et al. 2014), and it has been shown to result in localized water column enrichment (Amundrud et al., 2015). Procedural controls for deterrent were plaster blocks without carbaryl, and for nutrients were empty mesh bags. After four weeks, each plot was sampled for mesograzers density and epiphyte biomass, using methods outlined above (Seasonal surveys...). In addition to epiphyte dry mass, epiphytes were extracted in 90% acetone (W/V, at least 24 h in freezer) and measured for fluorescence (chlorophyll-a) by standard acidification method (Welschmeyer, 1994) on a Turner Designs 10-AU fluorometer. For chl-a, epiphytes were removed from one whole shoot in 2012 but from a consistent 15-cm portion above the sheath in 2011, which required less subsequent dilution to be within the range of detection by the fluorometer. In both cases, chl-a was standardized to the dry mass of *Z. marina* from which epiphytes were removed. The section of the shoot above the leaf sheath (y , g g⁻¹) provides a slightly elevated but robust indicator of whole-shoot epiphyte load (x , g g⁻¹, $y = 1.12x$, $r^2 = 0.93$, $n = 22$ shoots measured for epiphyte load in both ways from Jul. 2011 to Jul. 2012). Tissue from the youngest leaf of five shoots per plot was dried and packaged for nutrient analysis on a CHN analyzer (Thermo Fisher Scientific Inc., Waltham, MA; Duffy et al., 2015). The experiment ran from 19 June to 17 July 2011, and again from 1 August to 2 September 2012. Deterrent and control blocks were replaced twice each time.

For analysis, response variables of mesograzers density and biomass (sensu Edgar 1990), epiphyte chl-a and epiphyte dry mass, were standardized by *Z. marina* dry mass. Because mesograzers taxa could vary in their response to treatments, mesograzers density was also examined separately as isopods, caprellid amphipods, and gammarid amphipods. Tissue nitrogen content in *Z. marina* was included as a response variable to evaluate the effectiveness of nutrient addition treatments (2011 only). Linear models for these response variables were developed with

Table 2
Results, as F values (P values), of three-factor analysis of variance for response of crustacean mesograzers (summed, and by taxon), microalgal epiphytes, and tissue nutrients to crossed treatments of slow-release deterrent and nutrients in the water column. Two experimental trials were run under different growth and mesograzers conditions in different years, each with 10 replicate plots of each treatment. All response variables were standardized to dry biomass of *Zostera marina*.

Effect	Mesograzers abundance	Mesograzers biomass	Epiphyte chlorophyll-a	Epiphyte biomass
Deterrent	5.44 (0.02)	13.39 (0.0005)	3.02 (0.09)	0.48 (0.49)
Nutrients	6.02 (0.02)	4.78 (0.03)	1.40 (0.24)	0.19 (0.66)
Year	18.89 (<0.0001)	4.71 (0.03)	36.57 (<0.0001)	37.16 (<0.0001)
Det × nut	0.98 (0.32)	2.38 (0.13)	5.12 (0.027)	0.28 (0.60)
Det × year	0.26 (0.61)	0.02 (0.88)	0.004 (0.95)	0.016 (0.90)
Nut × year	1.72 (0.19)	0.81 (0.37)	1.12 (0.29)	1.13 (0.29)
Det × nut × year	0.006 (0.94)	0.25 (0.62)	0.009 (0.93)	0.39 (0.53)

Effect	Isopods (<i>Idotea resicata</i>)	Gammarid amphipods	Caprellid amphipods	Tissue nitrogen concentration
Deterrent	14.15 (0.0003)	2.33 (0.13)	1.56 (0.22)	3.12 (0.08)
Nutrients	0.71 (0.40)	2.13 (0.15)	4.71 (0.03)	3.59 (0.07)
Year	0.34 (0.56)	30.48 (<0.0001)	63.53 (<0.0001)	N/A
Det × nut	0.52 (0.47)	0.15 (0.70)	1.92 (0.17)	0.66 (0.42)
Det × year	0.026 (0.87)	1.38 (0.24)	1.58 (0.21)	N/A
Nut × year	0.018 (0.89)	2.33 (0.13)	6.35 (0.01)	N/A
Det × nut × year	1.83 (0.18)	0.23 (0.64)	0.52 (0.48)	N/A

Bold values indicate significance at $P < 0.05$.

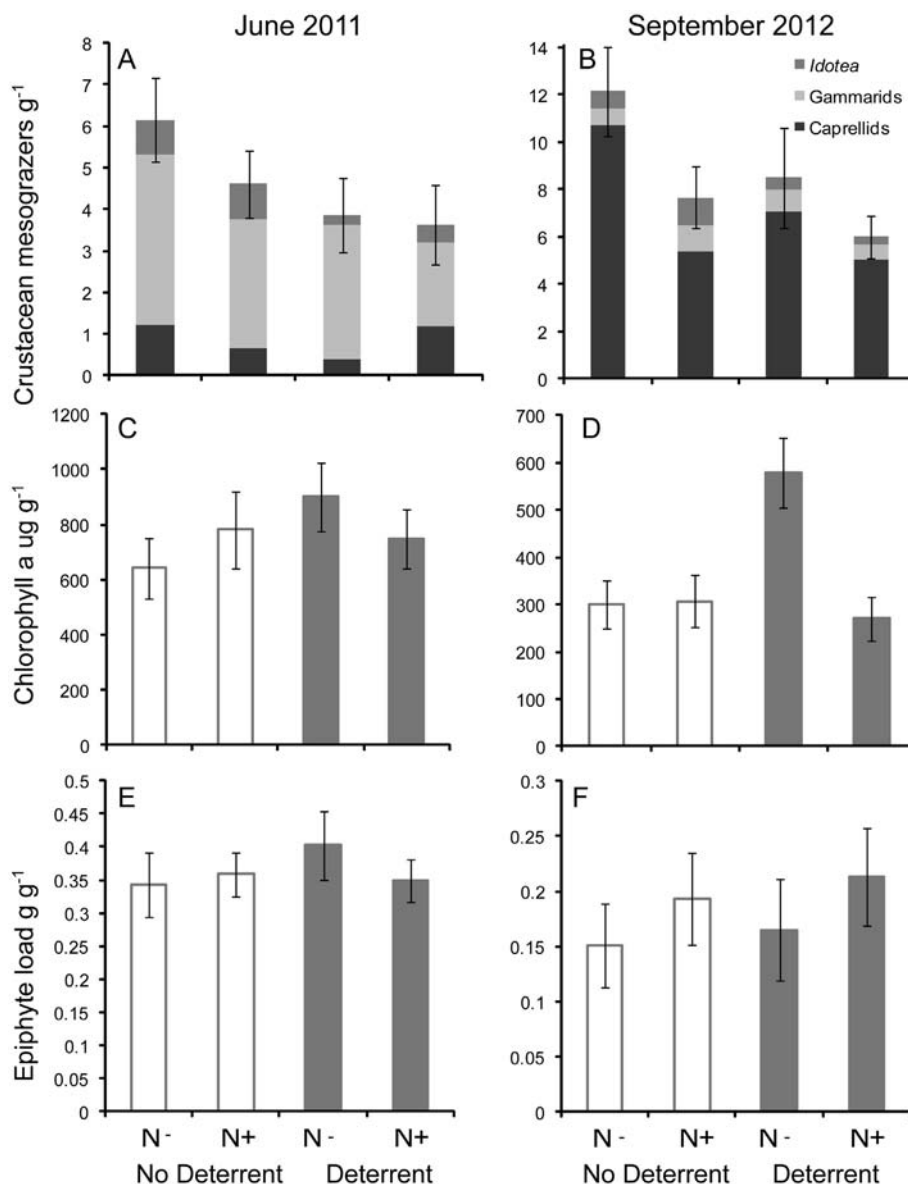


Fig. 2. Experimental response (mean \pm SE, $n = 10$) to mesograzer deterrent and water column nutrient addition (N+) in experiments run in June 2011 and September 2012. A, B) Crustacean mesograzers density, distinguished by dominant taxa, C, D) Epiphyte chlorophyll-a, and E, F) Total epiphyte dry mass, all standardized to dry biomass of *Z. marina*. Statistical results are in Table 2.

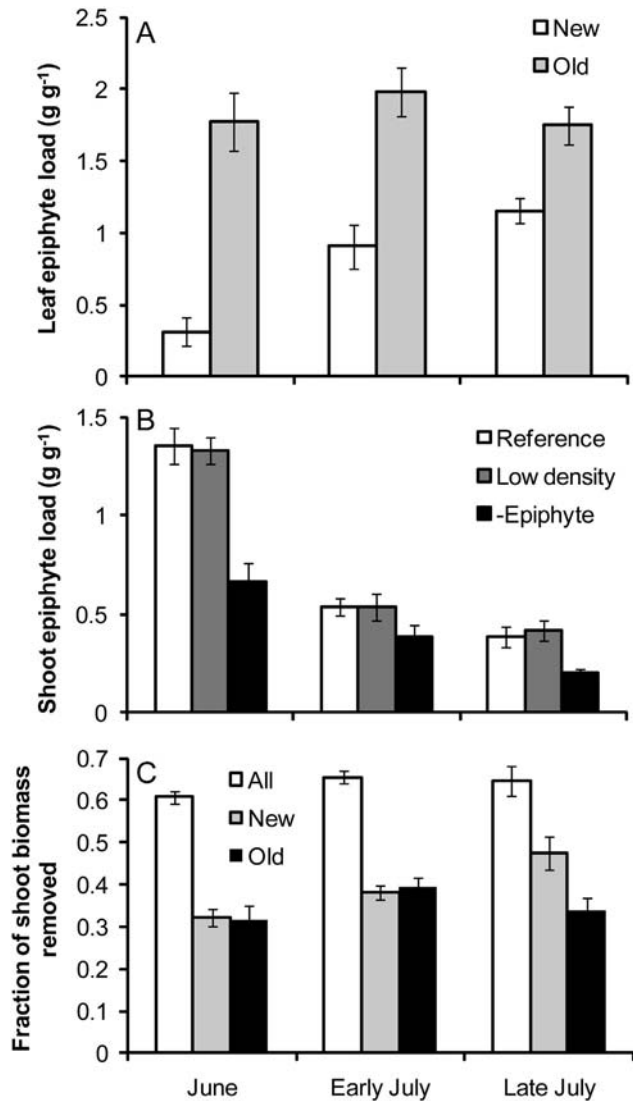


Fig. 3. Traits of *Zostera marina* shoots in three trials of experimental removal of epiphytes and leaves (mean \pm SE, $n = 10$). A) Epiphytes on new (inner three) or old (outer) leaves removed as part of initial treatments. B) Epiphytes on two treatments without cleaning (Reference, Low density) and one where epiphytes were removed 5–9 days previously (–epiphyte). C) Fraction of biomass removed when all, new, or old leaves were clipped at the top of the sheath.

mesograzers, deterrent, nutrient addition, and year as fixed effects, plus all two- and three-way interactions. Year was considered a fixed effect because eelgrass growth and mesograzers communities differ from June to August, but an approach that included Year as a random effect generated essentially identical statistical significance, so the results were not sensitive to the assumption of Year as a fixed or random effect.

2.4. *Zostera marina* growth response to epiphyte manipulations

Along an intertidal contour at MLLW, *Z. marina* shoots separated by at least 1 m were randomly assigned to one of six experimental treatments to evaluate shoot growth: 1) removal of epiphytes; 2) removal of all leaves above the leaf sheath; 3) removal of the three youngest leaves above the leaf sheath; 4) removal of all leaves except the three youngest above the leaf sheath; 5) removal of above-ground biomass around a target shoot for a distance of 15 cm; 6) reference shoots with no manipulation. This experiment, replicated with 10 shoots per treatment, was run three times through the summer of 2012 (25–30 June, 7–16 July, 23–30 July, for a total of 180 shoots).

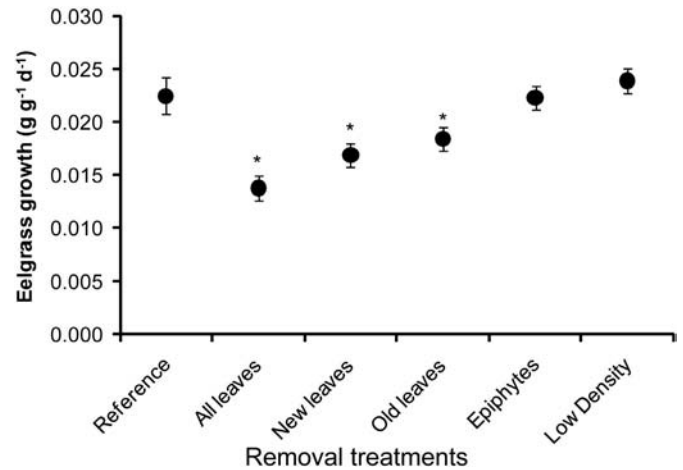


Fig. 4. *Zostera marina* specific growth under different experimental treatments of removing leaves above the sheath, removing epiphytes, or adjacent shoot removal (Low density), in comparison to Reference shoots. Points show means \pm SE from linear mixed-effect model results. Sample size was 10 replicates per treatment in each of three sequential trials in 2012. * $P < 0.05$ in difference from Reference.

Epiphyte removal (treatment 1, above) was accomplished in the field by placing each leaf on a flat plastic board and pulling a glass slide gently along the surface while the shoot remained intact and rooted in the sediment, a process that is unlikely to damage host tissue (Mazella and Alberte, 1986). For the treatments involving leaf removal (treatments 2–4), target leaves were clipped with the shoot still rooted. These clipped leaves were processed for epiphyte load, as above (Seasonal surveys...). Inner leaves are the youngest, most recently emerged leaves and are largely still extending, compared to the outer, older leaves that have already reached maximum extension.

In the field, each experimental shoot was identified by flagging tape on a heavy nail pushed into the sediment and was marked for shoot growth in a modification of the Zieman (1974) method. Two small

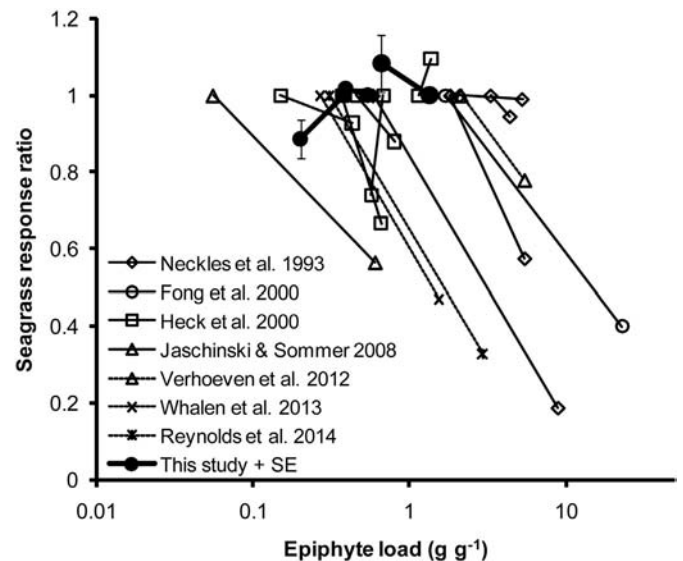


Fig. 5. Seagrass response to experimental variation in epiphyte load. Epiphytes were augmented through experimental increases in nutrients, reductions in grazers, or both, based on published literature. In contrast, epiphytes were directly reduced in this study. Seagrass responses (cover, area-specific growth, or mass-specific growth) were standardized as the response ratio in treatment relative to reference conditions (reference conditions shown on the y-axis as 1). Metrics for epiphyte load were converted to a common currency of g g⁻¹ using the following conversion factors: for epiphytes – 0.15 gAFDW/gDW (Neckles et al., 1993), 700 μ g chl-a/gDW (this study); for seagrass – 0.8 gAFDW/gDW (Westlake, 1974), 400 cm²/gDW (Ruesink et al., 2015, this study). See Supplemental Table 1 for data extracted from original published figures.

holes were made near the top of the leaf sheath with a sharpened wire. Marked shoots were collected after 5, 9, or 7 days in the sequential trials. Shoots were cut at the original mark in the leaf sheath in the field, so that no further leaf extension could influence the measurement of growth. Epiphytes were removed from each shoot and measured for dry biomass as described above (Seasonal surveys...). The original and newly-extended (between the cut and mark on growing leaves) portions of each leaf were measured, along with sheath width. Newly-extended portions were dried and weighed separately from the rest of the shoot for growth calculations, in which new biomass was divided by total biomass and by number of days between marking and collection.

Eelgrass response to treatments was determined through this metric of percent biomass increase per day, also called relative growth rate (e.g. Herbert and Fourqurean, 2009) or specific growth (e.g. Short et al., 1995). To calculate specific growth for leaf-removal treatments, the biomass of leaves removed at the beginning of the experiment was included in total biomass, which likely overestimated the biomass of older leaves, since some could have been lost naturally during the experiment. Consequently, a second growth metric was included that was not sensitive to the manipulations of biomass as part of setting up treatments: leaf extension per day, summed for all growing leaves on a shoot, with sheath width as a covariate. Experimental treatment (six levels) was a fixed factor, and trial date a random factor in linear mixed effects models for each response variable. Each treatment was evaluated for a change in growth rate relative to reference shoots.

3. Results

3.1. Seasonal patterns of epiphytes and mesograzers

Epiphyte load on *Z. marina* was best predicted by leaf emergence rate (Table 1), which had a parameter importance above 0.7 regardless of how mesograzers were included as a predictor, whether as total mesograzers or particular taxa. When leaves are being produced (and turned over) faster, the expectation is that epiphytes would also turn-over and be negatively related to leaf emergence rate; therefore, the positive relationship (Fig. 1A,D) is likely due to shared bottom-up control, although neither daylength (parameter importance ~0.2) nor temperature (parameter importance <0.02) by itself predicted epiphyte load as well as *Z. marina* leaf extension rate (Fig. 1B). Relative to above-ground dry mass of its eelgrass host (Fig. 1C), epiphyte load ranged from <10% in winter to ~60% in late spring (Fig. 1A). Mesograzers fluctuated in density more rapidly than at seasonal scales and changed in composition, from an April peak dominated by gammarids to a September peak of caprellids (Fig. 1E). Neither the taxon-specific nor total density of mesograzers helped explain seasonal variation in epiphyte load (parameter importance <0.15, Table 1). Shelled gastropods were rarely observed, but the shell-less opisthobranch *Phyllaplysia taylori* was present in September through April.

3.2. Epiphyte response to mesograzers and nutrient manipulations

The deterrent treatment successfully reduced crustacean mesograzers density by 29%, but nutrient addition also had a statistically significant main effect in reducing mesograzers density (30%; Table 2, Fig. 2A). Mesograzers biomass varied by treatment in the same way, reduced 39% and 25% by deterrent and nutrients, respectively (data not shown). Considering taxon-specific responses, isopods declined under deterrent, gammarid amphipods varied by year only (because less abundant in September than June), and caprellid amphipods were reduced by nutrient enrichment in 2012 (Table 2). Nutrient addition did not modify leaf nitrogen content (Table 2). Epiphyte chl-a was largest in the treatment with deterrent but without nutrients (deterrent \times nutrient interaction in Table 2, Fig. 2B). However, for

epiphyte load on a biomass basis, no treatments were distinguishable (Table 2, Fig. 2C).

3.3. *Zostera marina* growth response to epiphyte manipulations

Epiphytes accumulated with leaf age, as demonstrated by an epiphyte load on inner leaves that was 40% of epiphyte load on outer leaves (Fig. 3A). Removing epiphytes in the field was effective, with about half the epiphyte load on scraped vs. reference shoots at the end of the 5–9 day trials (Fig. 3B). The removal of leaves above the sheath reduced shoot biomass by 64%, evenly distributed between the three inner, new leaves and outer, older leaves (Fig. 3C). In comparison to reference shoots, *Z. marina* grew slower, as percent biomass per day, when new, old, or all leaves were removed, but neither epiphyte removal nor removal of nearby shoots influenced growth (Fig. 4). The results for leaf extension standardized to sheath width were similar except in one treatment: removal of old leaves reduced specific growth, but the effect was not statistically-significant for leaf extension due to higher variability (data not shown). Overall, removal of 64% of biomass reduced specific growth from 2.3% to 1.4% d⁻¹, which is a fractional reduction of only 39%. The effect of removing all leaves (–0.87%) was similar to the sum of the effects of removing new or old leaves (–0.97%; Fig. 4).

4. Discussion

4.1. Causes of epiphyte load on seagrass

Results from this study contribute to a growing body of literature comparing top-down and bottom-up control in seagrass ecosystems (Heck et al., 2000; Jaschinski and Sommer, 2008, 2010; Verhoeven et al., 2012; Whalen et al., 2013; Reynolds et al., 2014; Amundrud et al., 2015; Duffy et al., 2015). Mesograzers deterred indirectly increased epiphytes measured as chl-a, particularly when nutrients were not added (Fig. 2). This top-down effect may have been mediated primarily by isopods, which were the only taxon that declined significantly with deterrent (Table 2) and have rapid per capita grazing in seagrass systems (Williams and Ruckelshaus, 1993; Best and Stachowicz, 2012). Although nutrients appeared not to promote epiphytes in the present study, additions of water-column nutrients in the field frequently cause little response in epiphytes (Williams and Ruckelshaus, 1993; Verhoeven et al., 2012; Reynolds et al., 2014; Duffy et al., 2015). Instead, positive relationships between epiphytes and nutrients are typical in mesocosm studies with finer control over nutrient concentrations (Neckles et al., 1993; Williams and Ruckelshaus, 1993; Short et al., 1995; Moore and Wetzel, 2000; Jaschinski and Sommer, 2008). Nutrient loading may have been particularly ineffective in the present case because of naturally high nitrogen availability. In contrast, fertilization via slow-release nutrients in the water column stimulated both seagrass and epiphytes in the oligotrophic Florida Bay, and gastropod grazers showed little ability to exert top-down control (Peterson et al., 2007).

Observational and experimental approaches gave different insight into the mechanisms underlying epiphyte load, because epiphytes responded to experimental manipulations (Fig. 2) but showed no relationship to mesograzers over time (Fig. 1). In combination, however, the experiment suggests that epiphyte loads observed in summer could be still greater without top-down control, with the caveat that seasonal variation in epiphyte load was evaluated through dry mass, a metric that did not respond to the experimental manipulations. Other nearby regional studies of epiphyte loads on *Z. marina* show similar seasonal ranges to that identified for the Willapa Bay, i.e. 10–70% of host biomass on a dry weight basis. Macroalgal epiphytes peak in June on intertidal *Z. marina* in the Padilla Bay (Thom, 1990), and microalgal epiphytes peak in autumn on subtidal eelgrass in the San Juan Islands (Nelson and Waaland, 1997). These differences in structural and

functional aspects of seagrass epiphytes within one state support localized drivers of epiphyte load.

4.2. Consequences of epiphyte load on seagrass

In experimental evaluations of the consequences of epiphyte load, negative effects on seagrass have generally emerged following manipulations that augment epiphytes. This statement is well illustrated by the representative examples displayed in Fig. 5, which show negative relationships between seagrass productivity and epiphyte load despite a wide range of seagrasses examined, and despite inconsistent metrics for dependent and independent variables (but see Cook et al., 2011). Augmented epiphyte loads were achieved in these experiments by adding nutrients, removing grazers, or both. In contrast, in the present study, based on a direct test via epiphyte removal, competition between epiphytes and seagrass was not detected. As displayed in Fig. 5, this removal underestimates the actual manipulation, since epiphyte load was measured after 5 to 9 days of regrowth (Fig. 3B). In all three trials in the Willapa Bay, epiphyte load was on the low end of where seagrass productivity has been negatively affected (Fig. 5). At the same time, summer epiphyte loads in Willapa Bay are apparently rather high relative to standing levels in other estuaries, for instance, highest of 15 sites worldwide in the *Zostera* Experimental Network (Duffy et al., 2015).

Although high epiphyte loads can clearly impair seagrass productivity (Fig. 5), it does not yet appear possible to set a universal level of concern. Some of this uncertainty results from different ways of measuring epiphyte load, but also seagrass may have tolerance mechanisms. A key gap in understanding epiphyte effects is incorporating epiphyte distribution across host tissue, because epiphytes accumulate as leaves age (Bulthuis and Woelkerling, 1983; Mazella and Alberte, 1986; Borum 1987). Accordingly, the removal of the three youngest leaves, which were also least epiphytized (Fig. 3A), slowed plant growth (Fig. 4). It appears likely, then, that these fresh leaves have sufficient time before the build-up of epiphytes to contribute to whole-plant photosynthesis, and reliance on photosynthesis of rapidly-extending leaves provides a mechanism to tolerate epiphytes on the rest of the shoot. Because the removal of older, outside leaves slowed growth – though somewhat less than in other leaf-removal treatments – these leaves still apparently benefit whole-plant photosynthetic balance, rather than primarily competing with newer leaves. This contribution may stem from newly-extended tissue at the base of these leaves or from super-saturating light levels for this intertidal eelgrass population. Plant growth response to loss of even 30% of shoot biomass was rapid (Fig. 4, see also Ruesink et al., 2012, but see Cebrián et al., 1998), demonstrating the value of specific growth as an indicator of the integrated status of a shoot. Although thinning did not improve shoot growth, prior work demonstrated that thinned shoots invest more in asexual reproduction through branching (Ruesink et al., 2012), so shoot- as well as leaf-level productivity may be a more holistic indicator in longer-term studies.

The extent to which epiphytes at ambient levels reduce seagrass productivity can be inferred from bio-optical models (Zimmerman et al., 2015) but would benefit from additional direct reductions of epiphytes in the field, ideally involving more than two epiphyte loads so that any non-linearities in the relationship are revealed. The context for weak microalgal competition in the Willapa Bay could include a high-light intertidal habitat, nutrient-rich source water at low risk of eutrophication, and intact food web, all contributing to rapid leaf turnover. A point of concern should be that, when epiphyte loads begin to slow seagrass growth, they also increase the duration of leaves on the shoot and therefore allow further build-up of epiphytes on aging tissue. This potential reinforcing feedback could lead to high sensitivity of seagrass to small increases in the rate of epiphyte accumulation. Either weakened top-down or stronger bottom-up control could shift the epiphyte-seagrass relationship beyond a tipping point where seagrass could no longer out-grow its competitors.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jembe.2016.03.022>.

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