



Fish grazing enhanced by nutrient enrichment may limit invasive seagrass expansion

Fee O.H. Smulders^{a,*}, S. Tatiana Becker^a, Justin E. Campbell^b, Elisabeth S. Bakker^{d,e}, Mickey J. Boåsson^a, Mark M. Bouwmeester^a, J. Arie Vonk^{c,1}, Marjolijn J.A. Christianen^{a,1}

^a Wageningen University & Research, Aquatic Ecology and Water Quality Management Group, P.O. Box 47, 6700 AA, Wageningen, The Netherlands

^b Department of Biological Sciences, Institute of Environment, Florida International University, Miami, USA

^c Department of Freshwater and Marine Ecology (FAME), Institute for Biodiversity and Ecosystem Dynamics (IBED), University of Amsterdam, Amsterdam, The Netherlands

^d Department of Aquatic Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, The Netherlands

^e Wildlife Ecology and Conservation Group, Wageningen University, The Netherlands

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ABSTRACT

The success of invasive macrophytes can depend on local nutrient availability and consumer pressure, which may interact. We therefore experimentally investigated the interacting effects of nutrient (nitrogen and phosphorus) addition, the exclusion of large herbivorous fishes and mimicked grazing on the expansion rates of the invasive seagrass *Halophila stipulacea*. The experiments were established on Bonaire and Aruba, two islands in the southern Caribbean, which differ in fish community structure. We observed that multiple Caribbean fish species feed on *H. stipulacea*. At both study sites, nutrient enrichment decreased invasive leaf carbon:nitrogen ratios. However only on Bonaire, where herbivore fish abundance was 7 times higher and diversity was 4.5 times higher, did nutrient enrichment result in a significant reduction of *H. stipulacea* expansion into native *Thalassia testudinum* meadows. This effect was likely due to increased herbivory on nutrient enriched seagrass leaves, as we found that excluding large herbivorous fish (e.g. parrotfish) doubled invasive expansion rates in bare patches on Bonaire. On Aruba, *H. stipulacea* expansion rates were higher overall, which coincided with lower abundances and diversity of native fishes, and were limited by mimicked fish grazing. We suggest that top-down control by the native fish community may counteract eutrophication effects by increased grazing pressure on nutrient-rich invasive seagrass leaves. We conclude that diverse and abundant herbivore communities likely play an important role in limiting invasion success and their conservation and restoration may serve as a tool to slow down seagrass invasions.

1. Introduction

Coastal ecosystems around the world are experiencing a rapid increase in the number of non-native species that often negatively impact ecosystem functioning (Gallardo et al., 2016; Molnar et al., 2008; Papacostas et al., 2017). The success of invasive macrophytes may depend on local conditions such as nutrient availability and native biodiversity (Chapman, 2017; Havel et al., 2015). High nutrient availability may make a habitat more vulnerable to invasion (Gennaro et al., 2015; Wersal and Madsen, 2011), while high species richness and functional group diversity of the native plant and herbivore community

may enhance the resilience of an ecosystem via mechanisms of niche complementarity, competition and strong top-down control, thereby providing biotic resistance to invasions (Blowes et al., 2020; Elton, 1958; Gray et al., 2016; Polunin et al., 1993).

Consumer pressure is suggested to be the most important factor in limiting invasion success in both freshwater and marine ecosystems (Alofs and Jackson, 2014; Papacostas et al., 2017). Preference of native herbivores for invasive macrophytes can be site and species specific (Morrison and Hay, 2011; Tomas et al., 2010). Specialized herbivores preferring native plants can lead to invasive plants escaping top-down control (Christianen et al., 2019; Davis et al., 2005; Gollan and

* Corresponding author.

E-mail address: Fee.smulders@wur.nl (F.O.H. Smulders).

¹ These authors contributed equally (shared last authorship)

Wright, 2006; Vermeij et al., 2009b), following the ‘enemy release hypothesis’ (Keane and Crawley, 2002). However, since most marine herbivores are considered generalists (Fleming and Dibble, 2014; Parker and Hay, 2005), there are various examples of native generalist herbivores reducing invasive macrophyte success (e.g. Ribas et al., 2017; Tomas et al., 2011).

The impact of herbivores on invasion success can be altered by bottom-up forcing (Bakker et al., 2016; Gallardo et al., 2016; Wang et al., 2019). Local nutrient enrichment can increase survival and growth rates of fast-growing (invasive) plant species (Davis et al., 2000; Vermeij et al., 2009a; Wersal and Madsen, 2011), thus stimulating invasion. Invasive macrophytes that are adapted to quickly take up nutrients and invest their energy in expansion can therefore outcompete slow-growing native species under nutrient enriched conditions (Ceccherelli and Cinelli, 1997; Gennaro et al., 2015; Teixeira et al., 2017; Uddin and Robinson, 2018). However, fertilization may also alter the nutritional quality and chemical defenses of plants, potentially increasing the grazing pressure of (generalist) herbivores on nutrient-enriched leaves and their epiphytes (Bakker and Nolet, 2014; Campbell et al., 2018; Chase and Knight, 2006; Jiménez-Ramos et al., 2017; Liu et al., 2021), thus reducing invasion success. Depending on herbivore preference and invasive plant growth strategy, nutrient enrichment may favor either native plants or invasive plants. Thus, overall invasion success is likely dependent upon various site idiosyncrasies (Blumenthal, 2006).

Seagrass meadows form complex ecosystems harboring multiple seagrass species and associated herbivores (Heck and Valentine, 2006). Native tropical climax seagrass species are often adapted to oligotrophic conditions and high grazing pressure, by having (1) a complex root structure to access belowground nutrient pools, and (2) high levels of carbohydrate storage in the rhizomes (van Tussenbroek et al., 2006). Currently, seagrass meadows are prone to eutrophication, overfishing and invasion by exotic fast growing macrophytes (Waycott et al., 2009; Williams, 2007). The introduction of the invasive seagrass *Halophila stipulacea* to the Caribbean in 2002 led to reports of apparent competition and replacement of the native species *Thalassia testudinum* and *Syringodium filiforme* across multiple islands in the Caribbean (Winters et al., 2020).

Halophila stipulacea has been reported to invade both native seagrass habitat or previously unvegetated habitat (Smulders et al., 2017; Steiner and Willette, 2015; Willette et al., 2020; Willette and Ambrose, 2012). It is currently unknown whether native herbivore species graze on *H. stipulacea*, and what effects fish grazing and nutrient enrichment have on *H. stipulacea* expansion (Winters et al., 2020).

Understanding the relative importance of top-down and bottom-up effects on the expansion capacity of invasive seagrass is vital for the conservation of native seagrass meadows that are currently facing multiple threats. Here, we address the following questions: (1) Does nutrient enrichment impact *H. stipulacea* leaf nutrient content and expansion rates? (2) Does fish grazing affect the expansion rate of *H. stipulacea*? (3) How do nutrient enrichment and (mimicked) fish grazing interact to impact the expansion rates of invasive *H. stipulacea*? To answer these questions, we set up experiments at two islands in the southern Caribbean that likely differed in their top-down strength due to contrasting protection status: a protected seagrass meadow on Bonaire and an unprotected meadow on Aruba. Fertilization, mimicked fish grazing and enclosure treatments were used to assess the separate and combined impact of fish grazing pressure and nutrient availability on the expansion of *H. stipulacea*. We hypothesize that (1) nutrient enrichment will increase the leaf nutrient content at both sites but will only lead to an increase in expansion rates of invasive *H. stipulacea* on Aruba with lower fish densities compared to Bonaire, (2) fish grazing reduces the expansion rates of the invasive seagrass *H. stipulacea*; excluding herbivorous fish will increase invasive seagrass expansion rate on Bonaire where herbivorous fish density and diversity is high, while mimicked grazing will decrease invasive seagrass expansion rate on Aruba, where herbivore fish density and diversity is low, and (3) that

nutrient enrichment will strengthen the top-down control of herbivore fish on invasive seagrass when herbivore fish density and diversity is high (e.g. on Bonaire, but not on Aruba).

2. Materials and methods

2.1. Study species

In this study, we measured the responses of two seagrass species to bottom-up and top-down impacts. The native seagrass species *Thalassia testudinum* is a slow-growing climax species that is adapted to oligotrophic conditions (van Tussenbroek et al., 2006). A dense rhizome and root complex with stored carbohydrates ensures the ability of *T. testudinum* to withstand periods of intensive grazing (Christianen et al., 2019; van Tussenbroek et al., 2006). *Thalassia testudinum* generally shows year-round productivity in tropical areas, mainly expanding through clonal growth with limited new recruitment through seed dispersal (Tussenbroek et al., 2014). The seagrass species *Halophila stipulacea* is native to Red Sea, Persian Gulf and Indian Ocean, and spread consecutively to the Mediterranean Sea (first reported in 1894) and Caribbean Sea (first reported in 2002) (Ruiz and Ballantine, 2004; Winters et al., 2020). *Halophila stipulacea* is a fast-growing pioneer species capable of growing in high nutrient environments (Beca-Carretero et al., 2020; Helber et al., 2021; Winters et al., 2020). The species has wide phenotypic plasticity, potentially contributing to its invasive success (Winters et al., 2020). The roots of *H. stipulacea* are thin and expand quickly in the upper layer of the sediment (Smulders et al., 2017), which likely results in lower resistance to periods of intensive grazing. As of yet, there are no reports of sexual reproduction of *H. stipulacea* in the Caribbean Sea, and the species is assumed to use clonal growth as main reproductive strategy (Smulders et al., 2020). In native habitat with seasonal differences, *H. stipulacea* growth rates have been found to change across seasons (Beca-Carretero et al., 2020; Wahbeh, 1984). In invaded habitats without seasonal differences (as Bonaire and Aruba), *H. stipulacea* cover and growth rates appear consistent throughout the year (pers. obs. F.O.H. Smulders, S.T. Becker).

2.2. Study site

The experiments were set-up in two islands in the Southern Dutch Caribbean at the same latitude (12°N, no seasonality in sea temperature and sun hours), with contrasting protection status. On Bonaire, the experiments were conducted in Lac Bay (12.103261°N, 68.2390056°W) from October 2018 - December 2018 and October 2019 - November 2019. Lac Bay, located on the southeastern side of the island, is part of the Bonaire National Marine Park and the oldest marine reserve in the world (established in 1979) as well as a protected Ramsar site (Debrot et al., 2012). On Aruba, the experiments were conducted near the southwestern shore of the island in Barcadera (12.4802380°N, 69.9921950°W) from April 2018 - June 2018. The coastal zones of Aruba have historically been unprotected, with an increase in number of small scale fisheries over the past decades (Sadovy, 2005).

Lac Bay on Bonaire is an oligotrophic bay with extensive seagrass meadows (~ 7 km²) fringed by a coral reef. The invasive seagrass *H. stipulacea* has spread throughout the center of this bay since 2010 (Christianen et al., 2019). Grazing pressure is high within the bay, both by mega- and mesoherbivores. Megaherbivore green turtle (*Chelonia mydas*) densities are highest at the northeastern side and center of the bay resulting in either monospecific *H. stipulacea* meadows or mixed meadows with low-canopy native *T. testudinum* (Christianen et al., 2019). Mesoherbivore fish densities are highest in the shallow parts of the bay (e.g. the mangrove fringe) where absence of turtle grazing leads to monospecific *T. testudinum* meadows with a high canopy which provide food and shelter to a high diversity of fish (Debrot et al., 2012), which is constant throughout the year (unpublished results). Grazing pressure by epifaunal crustacean and invertebrate grazers is limited in

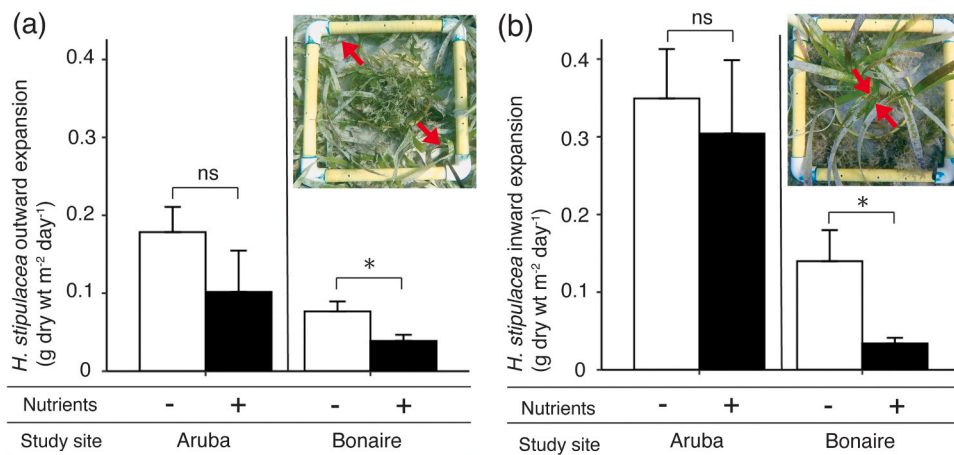


Fig. 1. Effects of nutrient enrichment on the expansion of invasive *Halophila stipulacea* in native seagrass habitat on Aruba compared to Bonaire ($n = 5$). Bars represent expansion rates ($\text{g dry wt m}^{-2}\text{day}^{-1}$; $\text{Avg} \pm \text{SE}$) of *H. stipulacea* (a) outwards of its transplantation sod into the surrounding native *T. testudinum* patch, and (b) into a transplantation sod of native *T. testudinum*. The arrows on the inset images depict the direction of *H. stipulacea* shoot expansion of the two separate transplantation experiments. Significant effects are indicated with an asterisk (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

this bay (unpublished results). Experimental plots were established near the mangrove fringe in monospecific meadows of the two seagrass species, where turtle grazing was absent (pers. obs. F.O.H. Smulders).

On Aruba, *H. stipulacea* was first reported in 2013 (Willette et al., 2014) and in 2020 has spread to almost the entire leeward coast (pers. obs. S.T. Becker). The study site Barchadera is protected from wave action by narrow mangrove-reef islands and a sandbar. Turtle grazing is absent at this site and fish densities are low. The seagrass meadows consist of both mixed and monospecific meadows of *H. stipulacea* and *T. testudinum*. Experimental plots were established nearshore in monospecific meadows of the two seagrass species, with the *H. stipulacea* plots situated closer to the mangrove-reef islands relative to the *T. testudinum* plots. For the experimental plots, we chose shallow seagrass meadows on Bonaire and Aruba that were similar in density and depth.

2.3. The impact of nutrient enrichment on invasive seagrass expansion into native seagrass

To investigate bottom-up control on expansion of invasive seagrass into native seagrass patches, reciprocal transplantation experiments with nutrient treatments were conducted on Aruba and Bonaire. An experimental duration of 10 weeks was chosen to be able to measure the responses of the fast-growing *H. stipulacea* and the slow-growing *T. testudinum* to nutrient enrichment. At each site, ten 0.25 m^2 plots were set up in an invasive species meadow and ten 0.25 m^2 plots were set up in a native seagrass meadow with at least 2 m distance between plots. Half of the plots were subjected to nutrient enrichment ($n = 5$, randomly assigned), using slow-release fertilizer enclosed in fiberglass mesh bags and suspended in the water column at the center of the plot (300 g Osmocote, NPK 14:14:14, following Campbell et al., 2018). The other plots did not receive any fertilizer (ambient conditions). In each plot, we transplanted circular seagrass sods with a diameter of 15.3 cm and 15 cm depth of the native species to the invasive plots and vice versa. At each site, three replicate procedural control plots (0.25 m^2) were established in which native and invasive seagrass sods (diameter 15.3 cm) were transplanted into its own habitat, to assess the impact of transplantation on shoot survival.

The expansion of the invasive seagrass was determined by counting the number of shoots that expanded through clonal expansion outside of its transplant (outward expansion) or into the native transplant (inward expansion) after 10 weeks. To compare expansion of *H. stipulacea* in native habitat to expansion in bare substrate, expansion of *H. stipulacea* shoots within a $0.25 \times 0.25 \text{ m}$ quadrant surrounding the invasive transplant (Fig. 1a) or within the 15.3 diameter area of the native transplant (Fig. 1b) was converted to gram dry weight $\text{m}^{-2}\text{day}^{-1}$ by first multiplying the shoot count with the average biomass shoot⁻¹ of newly grown shoots per location, then dividing by area (either within 15.3 diameter sods or within the plot minus the sod surface) and by experimental days. The impact of the treatments on native seagrass was measured by measuring

existing shoot densities of the native seagrass in all plots at the start of the experiment and again after 10 weeks, expressing this difference as percentage of shoots ($(\# \text{ shoots end} - \# \text{ shoots start}) / \# \text{ shoots start} * 100$). Additionally, we tested if there was an impact of native shoot density on the expansion rates of invasive seagrass by testing the strength of the relationship between the number of native seagrass shoots at the start of the experiment and the number of invasive seagrass shoots that had expanded outward and inwards after 10 weeks.

2.4. The impact of nutrient enrichment and fish grazing pressure on the expansion of invasive seagrass into bare substrate

To determine the combined impact of nutrient enrichment and fish grazing pressure on invasive seagrass expansion in bare substrate, we set-up two experiments with a crossed design. For this experiment, a duration of 4 weeks was chosen to measure the response of the fast-growing *H. stipulacea* to the treatments. On Aruba, a total of twenty 0.25 m^2 plots were created in a seagrass meadow invaded by *H. stipulacea* with at least 2 m between the plots. In each plot, a sediment core of 15.3 cm diameter and 15 cm depth was taken at the start of the experiment, creating a gap in the plot of the same dimensions. The bare gap was filled with clean sediment from that site until the sediment level was equal to the surroundings. Four treatments were set up ($n = 5$ each, randomly assigned); nutrient enrichment, mimicked grazing pressure, a combination of nutrient enrichment and mimicked grazing or ambient conditions. The nutrient treatments followed the same methods as described above. Mimicked grazing treatments were created by bi-weekly clipping of all leaves at 2 cm above the sediment surface using scissors (Kirsch et al., 2002; Short and Coles, 2001). Initial grazing pressure surveys at both study sites indicated a minimal number of fish bites on *T. testudinum* leaves on Aruba (0.4 bites leaf⁻¹) compared to Bonaire (2.4 bites leaf⁻¹). Additionally, nutrient enrichment only caused an increase in bite marks on Bonaire (Appendix B). Because of the high natural fish grazing pressure on Bonaire, an extra treatment with fish enclosures was added to the experimental design to exclude all herbivores > 1.5 cm. Enclosures were made from plastic extruded mesh (mesh size 1.5 cm, height 50 cm) that was cleaned weekly.

After 4 weeks, the number of *H. stipulacea* shoots that had regrown into the bare area was counted. Expansion of *H. stipulacea* shoots was converted to gram dry weight $\text{m}^{-2}\text{day}^{-1}$ by first multiplying the shoot count with the average biomass shoot⁻¹ of newly grown shoots per location, then dividing by area (within the 15.3 diameter gap) and by number of experimental days. In two plots at each site, a bioturbator had settled within the bare patch, disturbing the sediment and seagrass growth. Therefore, these plots were removed from analysis, reducing the number of replicates for these treatments from five to four. After 10 weeks of treatment, a subsample of seagrass leaves was extracted for plant nutrient content. Seagrass was dried at 60°C , ground with mortar and pestle and analyzed on a C/N elemental

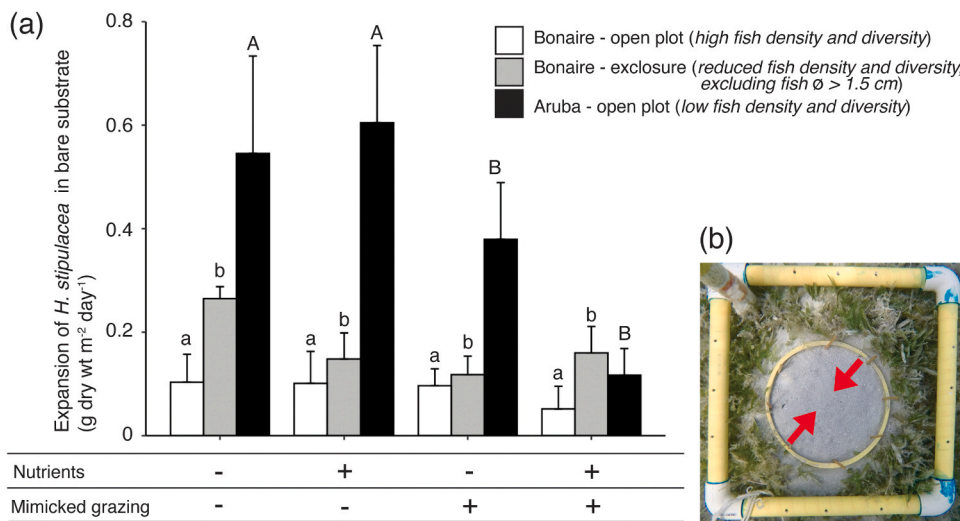


Fig. 2. (a) Effects of nutrient enrichment and mimicked grazing on expansion of invasive *Halophila stipulacea* into bare substrate on Bonaire with natural grazing (white), on Bonaire with fish enclosures (mesh size excluded fish with a diameter > 1.5 cm, grey), and on Aruba (black). Bars represent expansion rates in g dry wt m⁻² day⁻¹ (Avg ± SE n = 5). Statistical analysis was performed separately for Bonaire and Aruba. Significant differences between nutrient and (mimicked) grazing treatments are indicated with distinct lower-case letters for Bonaire, and distinct upper-case letters for Aruba (*P* < 0.05). (b) Arrows show the direction of expanded shoot biomass into bare substrate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Treatment effects on invasive seagrass expansion rates as analyzed with a three-factor ANOVA with the factors nutrient enrichment (N), caging (C) and mimicked grazing (G) on Bonaire and the two-factor ANOVA with the factors nutrient enrichment and mimicked grazing on Aruba. All factors had two levels. Shown are the df, Mean Square, F-values and P-values. Significant values are indicated in bold together with an asterisk (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

Site	Variable	df	MS	F	P value
Bonaire	Nutrients (N)	1	0.01115	1.065	0.3103
	Caging (C)	1	0.07396	7.065	0.0125 *
	Mimicked grazing (G)	1	0.02058	1.966	0.1712
	N x C	1	0.00121	0.115	0.7366
	N x G	1	0.00642	0.614	0.4396
	C x G	1	0.00368	0.352	0.5575
	N x G x C	1	0.02388	2.281	0.1414
Aruba	Nutrients (N)	1	0.0642	0.777	0.3931
	Mimicked grazing (G)	1	0.4749	5.743	0.0311 *
	N x G	1	0.1150	1.391	0.2579

analyzer (FlashEA 1112; Thermo Fisher Scientific, USA).

2.5. Fish community structure

The local fish community at each study site was determined using visual underwater surveys. A benthic area of 5 m x 2 m (10 m², 1 m height, n = 10 per site) was monitored while snorkeling. First, the observer waited for 5 min to minimize fish disturbance. Then for 10 min, the observer counted and identified all fish species in the array along a transect line. The last 5 min were used to move through the array and count fish hiding in the seagrass canopy. The observed number of fish and number of herbivore fish were calculated to individuals * 100 m⁻² (Polunin and Roberts, 1993).

Secondly, video analysis was used to observe which fish species

Table 2

List of seagrass herbivores observed on Bonaire and on Aruba during the visual and video surveys. Species with asterisk (*) were seen grazing on invasive *Halophila stipulacea* on video (appendix E).

Herbivore fish scientific name	Herbivore fish common name	Bonaire	Aruba	Literature supporting classification as seagrass herbivore
<i>Acanthurus chirurgus</i>	Doctor fish	X		(Greenway, 1976)
<i>Acanthurus coeruleus</i>	Blue tang	X		(Ogden, 1976)
<i>Cryptotomus roseus</i>	Bluelip parrotfish	X		(Dromard et al., 2017)
<i>Nicholsina usta</i>	Emerald parrotfish*	X	X	(Prado and Heck, 2011)
<i>Scarus iserti</i>	Striped parrotfish	X		(Dromard et al., 2017)
<i>Sparisoma aurofrenatum</i>	Redband parrotfish*	X	X	(Tribble, 1981)
<i>Sparisoma chrysopterum</i>	Redtail parrotfish*	X		(Lewis, 1985)
<i>Sparisoma radians</i>	Bucktooth parrotfish*	X		(Holzer et al., 2013)
<i>Sparisoma rubripinne</i>	Yellowtail parrotfish*	X		(Hay, 1981)

include *H. stipulacea* in their diet, and measure differences in fish density between plots with nutrient and enclosure treatments. A GoPro (Hero 3) with battery pack was attached to a PVC pole and positioned next to plots of the experiment assessing invasive seagrass expansion in bare areas, so all fish within the 0.5 m x 0.5 m plot were visible. 1-minute video fragments were sampled from a video of 3 h per treatment, with at least 30 min between each fragment to ensure independent replicates. During these 1-minute fragments, fish species and abundance were recorded following the MaxN method, to give a conservative estimate of relative density (Mallet and Pelletier, 2014). All fish species that were observed directly grazing on *H. stipulacea* were noted. At both study sites, fish density was compared between ambient and nutrient enriched plots. On Bonaire, fish density was additionally quantified in the enclosure treatments with or without nutrient enrichment.

2.6. Data analysis

All data was first checked for normality and homogeneity of variances (Shapiro Wilk test, Levene test, *P* > 0.05). Differences in native and invasive seagrass expansion with or without nutrient enrichment in the transplantation experiments were analyzed with an independent samples T-test, or Wilcoxon signed rank test as non-parametric alternative. A Pearson correlation coefficient was computed to assess the relationship between number of native shoots and number of invaded shoots. The expansion of invasive seagrass into bare patches and leaf C:N ratios were analyzed with a two-way ANOVA with mimicked grazing and nutrient enrichment as separate factors with each two levels on Aruba. For the Bonaire dataset, a similar procedure was followed using a three-way ANOVA with caging as a third factor with two levels. The factors were analyzed both separately as well as their interaction term. Differences in fish abundance between treatments were analyzed with a two-way ANOVA with caging and nutrient enrichment as separate factors with each two levels on Bonaire, and with a Wilcoxon signed rank

test comparing ambient and nutrient enriched plots on Aruba. Statistics were performed in R (R Core Team 2019) using the packages “dplyr”, “car”, “ggplot” and “ggpubr”. Average (Avg) values are presented together with standard errors (SE).

3. Results

3.1. The impact of nutrient enrichment on invasive seagrass expansion into native seagrass

Halophila stipulacea expanded both from the transplanted sods into the surrounding native meadows (outward expansion) and from the surrounding meadows into the transplanted native *Thalassia testudinum* sods (inward expansion) (Fig. 1). On Bonaire, nutrient enrichment caused a 2-fold reduction in outward expansion of *H. stipulacea* (T test, $t(8) = 2.5341, P < 0.05$, Fig. 1a) and a 4-fold reduction of the inward expansion of *H. stipulacea* into *T. testudinum* transplants (Wilcoxon test, $W = 24, P < 0.05$, Fig. 1b). On Aruba, the nutrient addition did not influence the outward or inward expansion of *H. stipulacea* ($P > 0.05$, Fig. 1).

There was no correlation between native shoot density at the start of the experiment and invasive seagrass expansion of both sites grouped together, both in the ambient plots ($r(17) = 0.30, P > 0.05$) and nutrient enriched plots ($r(17) = 0.21, P > 0.05$). The *T. testudinum* transplants did not expand but survived, at slightly lower densities on Aruba ($87 \pm 8\%$ and $86 \pm 4\%$ ambient and enriched) compared to Bonaire ($114 \pm 16\%$ and $110 \pm 6\%$ ambient and enriched), without significant differences between treatments ($P > 0.05$, Fig. C1). Native *T. testudinum* shoot number surrounding the *H. stipulacea* transplant did not show differences between nutrient enriched and ambient treatments in Bonaire and Aruba ($P > 0.05$, Fig. C2).

The seagrass shoots in the procedural controls showed on average $\geq 80\%$ survival for both species after 10 weeks: on Aruba for *H. stipulacea* $80 \pm 18\%$ and for *T. testudinum* $83 \pm 8\%$, and on Bonaire for *H. stipulacea* $82 \pm 3\%$ and for *T. testudinum* $90 \pm 5\%$.

3.2. Expansion of invasive seagrass into bare substrate

Inward *H. stipulacea* expansion into bare patches within their meadow was observed in all treatments within 4 weeks after clearing (Fig. 2). On Bonaire, excluding large fish (> 1.5 cm) doubled *H. stipulacea* expansion rates from 0.09 ± 0.02 – 0.18 ± 0.02 g dry wt m⁻² day⁻¹ (Three-way ANOVA, $F(1,30) = 7.065, P < 0.05$, Fig. 2a, Table 1). Highest average expansion rates were measured in enclosure plots without nutrients and clipping treatment (0.26 ± 0.02 g dry wt m⁻² day⁻¹). There was no significant effect of mimicked grazing or nutrient enrichment ($P > 0.05$) on expansion of *H. stipulacea* after 4 weeks.

On Aruba, mimicked grazing reduced *H. stipulacea* expansion rates compared to ambient grazing (0.25 ± 0.07 and 0.58 ± 0.11 g dry wt m⁻² day⁻¹ respectively, Two-way ANOVA, $F(1,14) = 5.743, P < 0.05$, Fig. 2a, Table 1). There was no significant effect of nutrient enrichment or interaction effect ($P > 0.05$) on *H. stipulacea* expansion.

Nutrient enrichment reduced C:N ratios in the aboveground biomass of *H. stipulacea* compared to ambient conditions, both on Bonaire (19.2 ± 0.7 – 16.3 ± 0.9 , Three-way ANOVA, $F(1,20) = 17.34, P < 0.001$) and Aruba (19.3 ± 0.7 – 16.6 ± 1.0 , $F(1,13) = 9.449$, Two-way ANOVA, $P < 0.01$, Fig. D1–4).

3.3. Fish community structure and grazing pressure

Underwater visual census revealed similar total fish densities between Aruba and Bonaire (472 ± 95 individuals 100 m^{-2} and 566 ± 113 individuals 100 m^{-2} respectively). However, herbivorous fish density was about 7 times higher on Bonaire (221 ± 51 individuals 100 m^{-2}) compared to Aruba (31 ± 12 individuals 100 m^{-2}). Total fish species richness was 28 on Bonaire and 14 on Aruba. On Bonaire, nine different herbivorous fish were encountered of which two were the only

herbivorous fish species observed on Aruba (Table 2).

In the video surveys the number of herbivorous fish observed on Bonaire was higher in the nutrient enriched plots compared to ambient plots (ambient: 0.5 ± 0.2 , enriched: 1.5 ± 0.6 fish min^{-1} , Two way ANOVA, $F(1,16) = 5.714, P < 0.05$). On Aruba, there was no significant effect of nutrient enrichment on observed fish densities (ambient: 0.0 , enriched: 0.4 ± 0.2 fish min^{-1} , Wilcoxon test, $P = 0.2$). Fewer fish were observed inside enclosures (0.4 ± 0.2 fish min^{-1}) compared to outside enclosures on Bonaire ($1.6, \pm 0.4$ fish min^{-1} , Two-way ANOVA, $F(1,16) = 8.229, P < 0.05$). All fish that were observed inside the enclosures were < 1.5 cm such as juvenile redband (*Sparisoma aurofrenatum*) and bucktooth (*Sparisoma radians*) parrotfish. The number of juvenile fish that still entered the cages was 0.2 ± 0.2 fish min^{-1} in ambient enclosure plots and 0.6 ± 0.4 fish min^{-1} in enriched enclosure plots. Several species were recorded to be grazing on *H. stipulacea* during video analysis (Table 2, Fig. E1–4).

4. Discussion

We document that the expansion of the invasive seagrass *Halophila stipulacea* in the Caribbean Sea may be limited by top-down control associated with herbivorous fishes. Bottom-up forcing through nutrient enrichment was found to enhance this top-down control in a habitat with a diverse and abundant fish community. This is further supported by our contrasting observations in a habitat with relatively low herbivore densities where *H. stipulacea* invasion rates were higher in general and only impacted by mimicked grazing. In both habitats, native *Thalassia testudinum* shoot density did not change with or without nutrient enrichment and native shoot density did not impact invasive expansion success, indicating that competitive interactions did not drive this effect. Our results provide novel insights into the relative and combined effect of bottom-up and top-down processes in invaded seagrass meadows.

4.1. Effects of nutrient enrichment

Nutrient enrichment can increase plant productivity and biomass, favoring fast-growing invasive macrophyte species over native species (Gennaro and Piazzini, 2011; Teixeira et al., 2017; Wersal and Madsen, 2011). In this study, we measured a reduction in *H. stipulacea* leaf C:N ratios within the nutrient enriched plots on Bonaire and Aruba, indicating increased nutrient uptake. However, instead of a corresponding increase in biomass production rates, we found a decrease on Bonaire and no effect of nutrient enrichment on Aruba. On Bonaire, this resulted in a 50% reduction in outward and 79% reduction in inward expansion rate under enriched compared to ambient conditions. This is in contrast to previous research where nutrient enriched conditions corresponded to high invasion success of fast growing macrophytes (Ceccherelli and Cinelli, 1997; Gennaro and Piazzini, 2011; Teixeira et al., 2017; Wang et al., 2019), including *H. stipulacea* (van Tussenbroek et al., 2016). A likely explanation for the changes in expansion of *H. stipulacea* under enriched conditions on Bonaire, is that the diverse and abundant fish community responded to nutrient enrichment by increasing grazing pressure while on Aruba the low-diverse community did not cause this effect. The same mechanism of increased grazing pressure after nutrient enrichment was found in our initial grazing surveys on Bonaire (Fig. B1, B2). Nutrient toxicity is a less likely explanation for this reduced expansion as pilot studies in the lab showed that *H. stipulacea* could withstand high nutrient concentrations in uptake experiments (unpublished results). In the short-term experiment assessing *H. stipulacea* expansion rates into bare substrate, no significant impact of nutrient enrichment was observed on Bonaire. However, in our video analysis, we did measure significantly higher fish abundance in the nutrient enriched plots, which may have been attracted to the enriched seagrass leaves and their epiphytes (Campbell et al., 2018; McSkimming et al., 2015; Mutchler and Hoffman, 2017). Nutrient enrichment increased the leaf nutrient content in our study, which has been found to enhance the

plant palatability to herbivores, increasing grazing pressure and potentially reducing expansion rates (Boyer et al., 2004; Campbell et al., 2018; Jiménez-Ramos et al., 2017; Prado and Heck, 2011; Ravaglioli et al., 2018). Although more research is needed into the long-term effects of nutrient enrichment on both native and invasive seagrass, we conclude that in systems with an intact generalist herbivore community, the interaction between local nutrient enrichment and biotic control may have an opposite impact on invasive seagrass success compared to systems with depleted herbivore communities.

4.2. Effects of fish grazing

Apart from nutrient-induced grazing effects, we confirmed our hypothesis that direct grazing had a significant negative impact on invasive plant expansion, both as natural fish grazing on Bonaire as well as mimicked grazing on Aruba. We showed that invasive seagrass expansion rates could be doubled by locally excluding fish > 1.5 cm on Bonaire and reduced by > 50% with mimicked grazing on Aruba. Mimicked grazing did not significantly impact expansion on Bonaire, likely because of background grazing pressure both in and outside of enclosure plots reducing the impact of this treatment, while on Aruba limited ambient grazing pressure caused a large treatment effect. Our results indicate that invasive seagrass expansion rates were higher on Aruba compared to Bonaire, while the herbivore community was 4.5 times as diverse and seven times more abundant on Bonaire compared to Aruba. Together with our experimental results, our study suggests that a diverse and abundant fish community may provide biotic resistance to invasive macrophyte species. Studies about grazing impacts of herbivores on invasive macrophyte expansion are limited, but can provide new insights into biotic resistance theory, since most aquatic herbivores are generalist feeders (Bakker et al., 2016; Petruzzella et al., 2017), and have been shown to select their food source based on plant traits instead of novelty (Grutters et al., 2017). Based on our study, nutrient-induced and direct grazing effects by an abundant herbivore community may even be more important in determining the competitive outcome than direct impacts of nutrients on plant physiology. Top-down control could therefore reduce the competitive advantage fast-growing invasive species might have in nutrient-rich conditions. In future experiments assessing fish grazing pressure using enclosures, it should be taken into account that herbivore fish < 1.5 cm can still have significant grazing impacts. Feeding trials are needed to reveal the preference of the various seagrass herbivores for native and invasive macrophytes, to further predict the potential of biotic resistance in invaded seagrass meadows (Parker et al., 2006).

4.3. Herbivore community

High grazer abundance and diversity may have contrasting effects on the expansion of invasive species depending on food preferences of the species (Engelen et al., 2011; Gollan and Wright, 2006; Tomas et al., 2011). Based on our results, the dense *T. testudinum* border close to the mangroves within the invaded area of Bonaire is likely more resistant to seagrass invasion compared to the center. High-canopy seagrass harbors greater fish abundance and diversity because of shelter and spillover from the mangroves (Debrot et al., 2012). Besides known generalist seagrass herbivores such as the bucktooth (*Sparisoma radians*) and red-band (*Sparisoma aurofrenatum*) parrotfish (Kirsch et al., 2002; Weinstein and Heck, 1979), the emerald parrotfish (*Nicholsina usta*) was often seen grazing on *H. stipulacea* on Bonaire. This species is increasing in number in the Gulf of Mexico due to tropicalization (Fodrie et al., 2010), was found to consume five to 36 times more native seagrass than other grazers in an earlier study (Prado and Heck, 2011), and may therefore be an important ecological component in biotic resistance in seagrass meadows. High herbivore fish density and diversity together with our experimental results help to understand the current absence of *H. stipulacea* in high-canopy *T. testudinum* habitat (Christianen et al.,

2019). In contrast, green sea turtles have been shown to prefer native seagrass and crop it short thereby facilitating invasive seagrass expansion in certain areas within the same bay (Christianen et al., 2019). This explains that even within a protected area with high biodiversity, invasive seagrass densities can become high. A dynamic grazing regime of green turtles resulting in variation in *T. testudinum* canopy height (Christianen, 2021) may sustain areas with high herbivore fish densities and therefore locally limit *H. stipulacea* expansion. Similarly, in Mediterranean seagrass habitats it has been previously suggested while some herbivore fish species may limit the expansion rate of invasive *Caulerpa racemosa*, the protection of marine habitat can also lead to returning herbivorous fish grazing on native seagrass leaves, therefore promoting the invasion of the alga (Caronni et al., 2015). The degree of biotic resistance to invasion is therefore species and context dependent (Caselle et al., 2018).

4.4. Protection status

A more diverse herbivore community likely has the potential to exert top-down control over the invasive seagrass species *H. stipulacea* and therefore decrease the success of these invaders. High (juvenile) herbivorous fish diversity and density may be related to the protection status of the habitat (Alonso Aller, 2018; Alonso Aller et al., 2017; Polunin et al., 1993). Even though the role of parrotfish in increasing the resilience of other marine ecosystems like coral reefs inside protected areas is being questioned due to lack of field data (Bruno et al., 2019), our results provide first evidence that high levels of parrotfish grazing pressure within the selected protected area most likely decreases the expansion rates of invasive seagrass. Apparent higher general expansion rates of the invasive seagrass on Aruba compared to Bonaire could be the result of low fish grazing pressure, however other factors related to protection status could also be important drivers. Concentrations of nitrogen in *H. stipulacea* and *T. testudinum* leaves were similar at both study sites, suggesting nutrient loading to be comparable in both habitats. However, in the unprotected area on Aruba, the impact of local anthropogenic disturbances (e.g. a drink- and wastewater processing facility), was reflected by a high algal cover (predominantly *Acanthophora spicifera*) and abundance of detritivores. This high algal cover on Aruba may shift feeding preferences of fishes to algae (Duarte, 1995; Littler et al., 2006), and because the algal layer is situated on top of the seagrass it can also physically prevent fish from foraging on the seagrass (Heck and Valentine, 2006). In future studies it would be valuable to include measurements of water quality and the response of algae, including epiphytes, to nutrient enrichment. Invasion success can be driven by a combination of different trophic effects, resilience of the native plant community, water quality, anthropogenic disturbance and many other mechanisms that should be considered when managing an area. Our study provides a starting point to investigate the relationship between marine protection status and biotic resistance to invasive seagrass species in seagrass ecosystems.

4.5. Conclusion

As demonstrated by the fast expansion and settlement of *H. stipulacea* to various islands in the Caribbean since 2002, the arrival and subsequent expansion of *H. stipulacea* to new habitats is often unavoidable (Smulders et al., 2017; Willette et al., 2020; Winters et al., 2020). Plant invasion can have unforeseen impacts on ecological interactions and ecosystem services in seagrass meadows (James et al., 2020; Williams, 2007). Therefore, it is important to maintain or even enhance seagrass resistance to invasion. A key role for limiting expansion of invasive plant species may be provided by the community of generalist herbivores, including fish species. Management strategies to maintain or restore these generalist herbivore communities and their habitats can therefore be important to mitigate potential negative effects of invasive plants after their establishment in new seagrass habitats.

CRedit authorship contribution statement

F.O.H.S. led the writing of the manuscript; F.O.H.S, M.J.A.C., J.E.C, J.A.V and S.T.B. conceived ideas and designed methodology; F.O.H.S., S.T.B, M.J.B. and M.M.B collected the data; F.O.H.S., S.T.B, M.J.B. and M.M.B analyzed the data and M.J.A.C. acquired funding for the project. All authors contributed critically to the drafts and gave final approval for publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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Appendix A

See

Table A1. Characteristics of the study sites on Bonaire and on Aruba as found in this study, with underwater pictures of the *H. stipulacea* meadows.

	Bonaire	Aruba
Protection status	Protected	Unprotected
<i>T. testudinum</i> shoot density (shoots/m ² ± SE)	304.6 ± 0.5	399.8 ± 0.6
<i>H. stipulacea</i> shoot density (shoots/m ² ± SE)	4939 ± 979	5898 ± 395
Depth (m)	1.5	0.7
Algae cover	Low	High
% DW N in <i>H. stipulacea</i> leaves	1.4 ± 0.09	1.4 ± 0.05
% DW N in <i>T. testudinum</i> leaves*	2.0 ± 0.13	1.8 ± 0.25
Nearby industrial human activities	Low	High

* Methods correspond to described methods of the determination of %N in *H. stipulacea* leaves

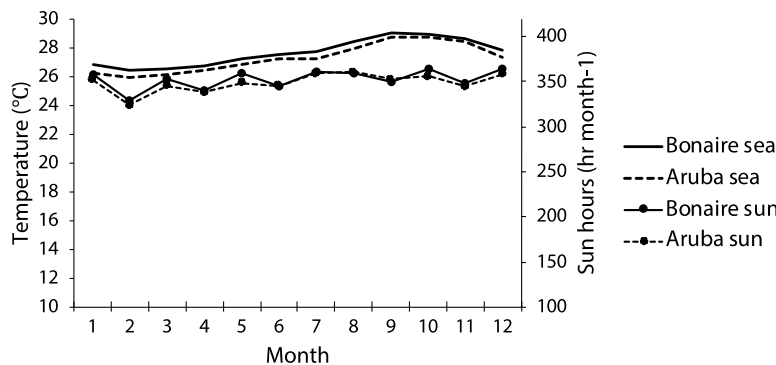
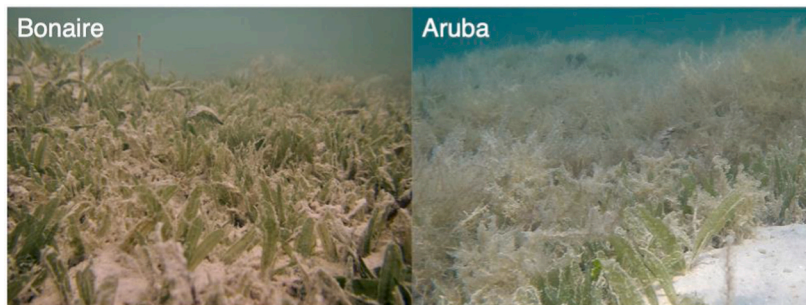


Figure A1. Historical averages of surface sea water temperature (<https://seatemperature.info/>) and sun hours (<https://www.worldweatheronline.com/>) per month on Bonaire and Aruba.

Appendix B

See Appendix Fig. B1–2.

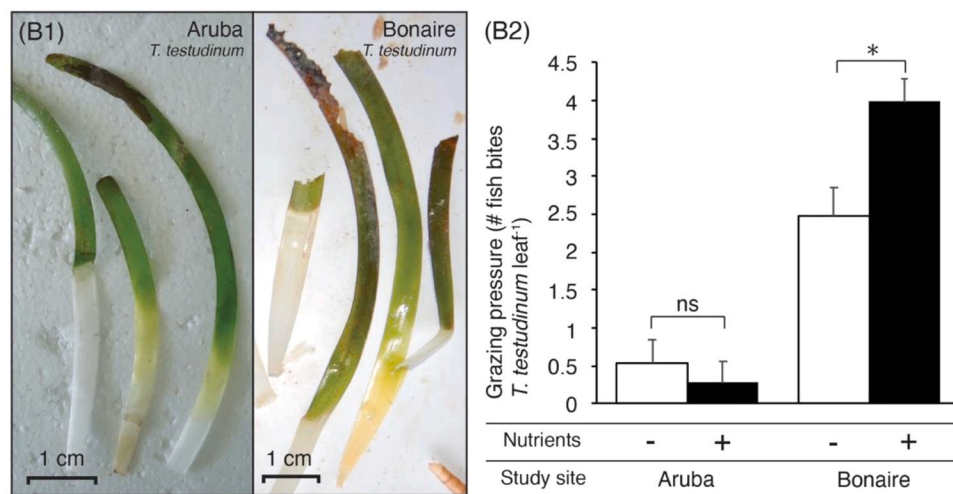


Fig. B1–2. Figures indicating low grazing pressure on Aruba compared to Bonaire (B1) Pictures of *T. testudinum* leaves sampled on Bonaire and Aruba. (B2) Comparison of grazing pressure on native seagrass leaves on Aruba and Bonaire with and without nutrient enrichment. Bars represent # fish bites per *Thalassia testudinum* leaf (Avg ± SE) based on 10 shoots per replicate (n = 5). Significant effects are indicated with an asterisk (Two-sample T test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Appendix C

See Appendix Fig. C1–2.

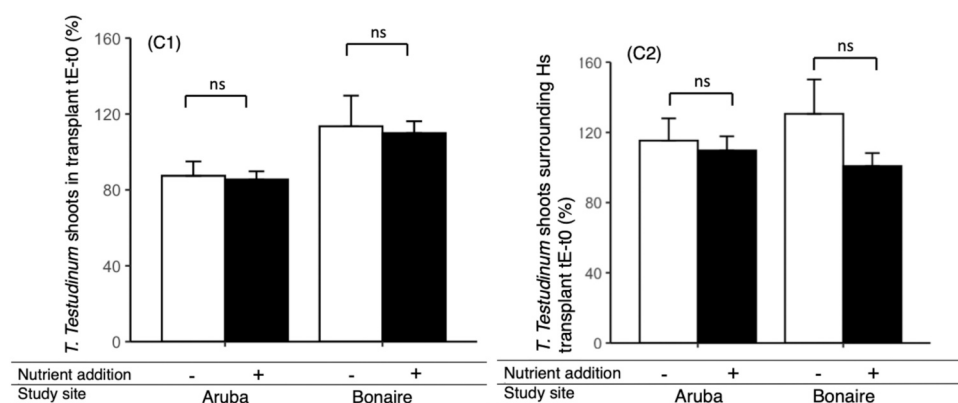


Fig. C1–2. Relative changes in *T. testudinum* density (%) over the course of the *H. stipulacea* expansion experiments (Avg ± SE) for the transplanted *T. testudinum* sods (panel) and the *T. testudinum* plots surrounding the *H. stipulacea* sods (right panel) in both ambient (white bars) and nutrient (black bars) treatments. Only small changes in *T. testudinum* were observed, while nutrient enrichment did not induce significant effects (n.s.).

Appendix D

See Appendix Fig. D1–2.

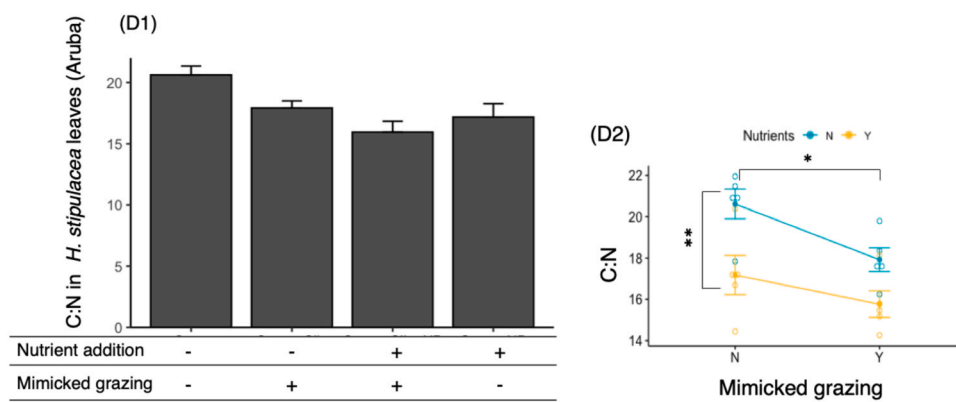


Fig. D1–2. Effects of nutrient enrichment and mimicked grazing on C:N ratios of *Halophila stipulacea* leaves regrown into bare substrate on Aruba (n = 5). (D1) Bars represent C:N ratios (Avg ± SE). (D2) Two-way interaction plot indicates significant differences between nutrient and mimicked grazing treatments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

See Appendix Fig. D3–4.

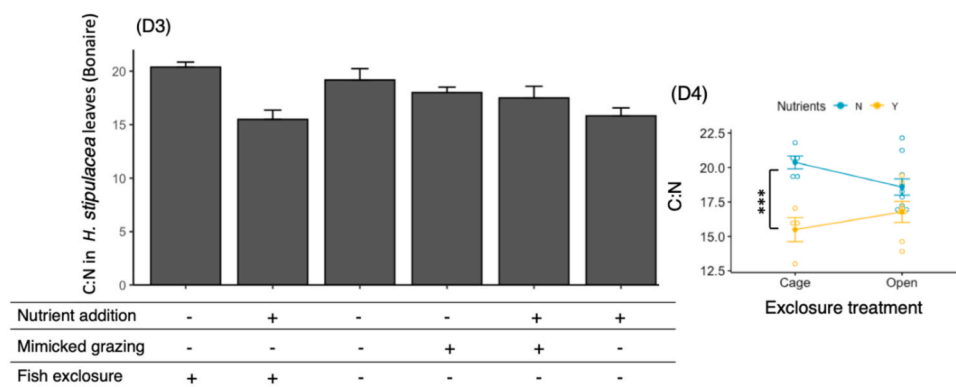


Fig. D3–4. Effects of nutrient enrichment, mimicked grazing and fish exclusions on C:N ratios of *Halophila stipulacea* leaves regrown into bare substrate on Bonaire (n = 5). (D3) Bars represent C:N ratios (Avg ± SE). (D4) Two-way interaction plot indicates significant differences between nutrient and exclusion treatments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Appendix E

See Appendix Fig. E1–4.

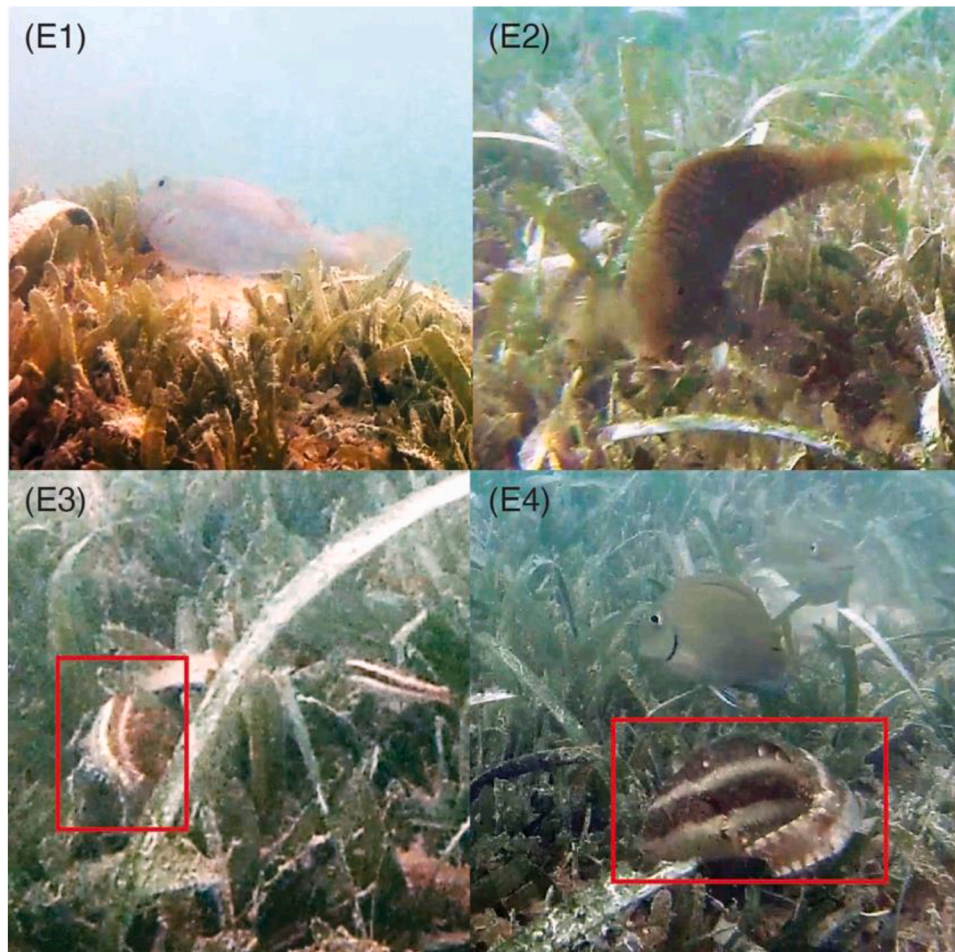


Fig. E1–4. Pictures 1–4: Screenshots from videos showing evidence of fish taking bites of *Halophila stipulacea* leaves on Bonaire. (E1) emerald parrotfish (*Nicholsina usta*). (E2) yellowtail parrotfish (*Sparisoma rubripinne*). (E3) bucktooth parrotfish (*Sparisoma radians*) (E4) redband parrotfish (*Sparisoma aurofrenatum*).

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