Simulated overfishing and natural eutrophication promote the relative success of a non-indigenous ascidian in coral reefs at the Pacific coast of Costa Rica

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Abstract

Colonial ascidians of the genus Didemnum are common fouling organisms and are typically associated with degraded ecosystems and anthropogenic structures installed in the sea. In this study, however, the non-indigenous ascidian Didemnum cf. perlucidum Monniot F., 1983 was discovered in coral reef environments on the Pacific coast of Costa Rica. Its role in the succession of a benthic community and the impact on biogeochemical features (i.e. reef cementation) was assessed by deploying terracotta settlement tiles on the reef for 24 weeks. Predator exclusion in experimental plots and naturally elevated nutrient concentrations during seasonal coastal upwelling gave insights on how settlers of D. cf. perlucidum succeed under projected environmental change. Exclusion of larger predators and grazers caused an increase of D. cf. perlucidum coverage on tiles from 7 to > 80%. Due to its rapid proliferation, D. cf. perlucidum grew over calcifying reef organisms, such as barnacles, polychaetes, and crustose algae, and significantly decreased the accumulation of inorganic carbon on the settlement tiles by one order of magnitude (4.6 to 0.4 mg C cm⁻²). The combination of reduced predation and eutrophication revealed negative synergistic effects on the accumulation of inorganic carbon. The opportunistic reaction of D. cf. perlucidum to environmental changes was further evident by 2-fold increased growth rates that were positively correlated (\(r^2 = 0.89\)) to seawater particulate organic matter (POM) concentration during coastal upwelling. These results suggest that D. cf. perlucidum is a strong spatial competitor in Eastern Tropical Pacific coral reefs that face changing environmental conditions, e.g. overfishing and eutrophication. The effects of this species on disturbed benthic communities, but also its potential role as a habitat modifier, is likely significant. Thus, a continuous monitoring of D. cf. perlucidum is recommended to better understand their effects on post-disturbance dynamics in coral reef ecosystems.

Key words: benthic community structure, biofouling, Didemnum perlucidum, settlement plates, recruitment, phase shifts

Introduction

Coral reefs are affected by regular, large-scale natural disturbances (e.g., tropical storms, bleaching events, or crown-of-thorn sea star outbreaks) that can considerably alter the benthic community structure (Lamy et al. 2016). However, in the absence of chronic anthropogenic stressors, resilient ecosystems are usually capable of recovering to pre-disturbed states (Jones and Schmitz 2009). After disturbance, bare reef substrate is successfully re-colonized by diverse benthic organisms, including crustose coralline algae (CCA) that may then facilitate the settlement of coral larvae and promote recovery (Ritson-Williams et al. 2010).

Human activities and associated changes in environmental conditions, however, play a major role in the post-disturbance dynamics, and may negatively affect primary (uncolonized substrate) and secondary (previously colonized surfaces) community succession
Coral reef surveys carried out at the Northern Pacific coast of Costa Rica have been increasingly threatened due to a high organic matter. Additionally, fish stocks in the same area have been increasingly threatened due to a high demand for local reef fish, with unknown effects for fish populations (Villalobos-Rojas et al. 2014) and the local benthic communities. During extensive coral reef surveys carried out at the Northern Pacific coast of Costa Rica in 2013 and 2014 (Roth et al. 2015; Stuhldreier et al. 2015b), a suspected non-indigenous Didemnidae succeed in natural reef systems and under changing environmental conditions. The present study assessed patterns of recruitment and growth of the ascidian Didemnum sp. in the succession of a natural benthic reef community on the Pacific coast of Costa Rica. Terracotta settlement tiles in open (control) and caged (simulated overfishing) experimental plots were deployed on a reef for 24 weeks (before and during seasonal coastal upwelling) to answer the following questions: 1) How do recruitment, growth, and the relative abundance of Didemnum sp. respond to simulated overfishing, natural eutrophication resulting from seasonal upwelling, and a combination of both simulated overfishing and natural eutrophication?; 2) How do these changes influence reef accretion through the accumulation of inorganic carbon?

Material and methods

Study area

This experiment was carried out from October 2013 until April 2014 (duration = 24 weeks) at a reef located in the Gulf of Papagayo on the Northern Pacific coast of Costa Rica (10°32′19″N; 85°45′54″W) (Figure 1A). The reef is dominated by the branching hard coral Pocillopora spp. with small patches of sand and extends at a water depth of 5–7 m (depending on the tides). The Gulf of Papagayo experiences seasonal wind-driven coastal upwelling that usually takes place between December and March (Jiménez 2002).

Environmental variables

A range of environmental variables (temperature, light availability, nutrient concentrations, chlorophyll a, particulate organic nitrogen (PON), particulate organic carbon (POC), and dissolved organic carbon (DOC)) were measured weekly at the study site. A detailed description of the sampling procedure and analytical methods can be found in Stuhldreier et al. (2015a). Briefly, water temperature and light availability were determined continuously with HOBO® Pendant (Onset Computer Corporation, Cape Cod, USA) temperature data loggers and a self-contained PAR (photosynthetically active radiation) logger with a planar cosine-corrected sensor (Odyssey Integrating PAR sensor, Dataflow Systems PTY Limited, Christchurch, New Zealand), respectively, and daytime averages were calculated. Water for the determination of all other variables was sampled in triplicates directly above the reef substrate. Water samples for inorganic nutrient concentrations were filtered through disposable syringe filters (pore size 0.45 μm).

Colonial ascidians have only recently caught attention in post-disturbance dynamics of coral reefs (e.g. Bak et al. 1996; Shenkar et al. 2008; Shmuel and Shenkar 2017), as they were often only associated with anthropogenic structures (e.g. ports, marinas, shellfish cultures). Nevertheless, once established in a new environment, even small populations may become a constant source of propagules that are capable of rapidly overgrowing benthic organisms (López-Legentil et al. 2015; Shenkar et al. 2008). By a combination of high growth rates and high reproductive outputs, these species may quickly outcompete native benthic organisms (Rius et al. 2009). However, the successful establishment of populations of invasive ascidians depends on the biological characteristics (e.g. food availability and predation pressure) of the new habitat (López-Legentil et al. 2005; Simkanin et al. 2013).

Coral reefs at the North Pacific coast of Costa Rica are exposed to highly dynamic environmental conditions. Seasonal coastal upwelling between December and March (Jiménez 2002) results in higher concentrations of inorganic nutrients and organic matter. Additionally, fish stocks in the same area have been increasingly threatened due to a high demand for local reef fish, with unknown effects for fish populations (Villalobos-Rojas et al. 2014) and the local benthic communities. During extensive coral reef surveys carried out at the Northern Pacific coast of Costa Rica in 2013 and 2014 (Roth et al. 2015; Stuhldreier et al. 2015b), a suspected non-indigenous colonial ascidian of the genus Didemnum was observed in many cryptic habitats of the reef, such as crevices and on the bases of coral colonies. Even though some studies have analyzed the effects of pollutants (e.g. Mayer-Pinto and Junqueira 2003) and predation pressure (e.g. Kremer and da Rocha 2016) on the succession of colonial ascidians on
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Figure 1. Map of the Pacific coast of Costa Rica (A) and illustrations of the experimental setup (B and C). The map (A) indicates the location of the study site Matapalo Reef at the Northwestern Pacific coast of Costa Rica. Experimental cages to simulate overfishing were randomly deployed on the reef (B). Graphical visualization of the experimental frames with settlement tiles (C). Control (left) and simulated overfishing treatment (right). Overfishing was simulated by covering assigned setups with mesh net with 2 cm diameter opening. Insert shows the side view of settlement tiles that were attached to the frames in a 45-degree angle relative to the substrate on a tough plastic net fixed between the vertical poles. MWD = Mean water depth. Photo by F. Roth.

into acid-washed 15 mL glasses (for ammonia NH_4^+ and phosphate PO_4^{3-}) or 50 mL polypropylene containers (for nitrate NO_3^-). NH_4^+ was determined fluorimetrically within 24 h after sampling (Trilogy® Laboratory Fluorometer, Turner Designs Inc., San Jose, USA) after overnight incubation with orthophthaldialdehyde-solution (OPA) in the dark (limit of detection (LOD) = 0.023 µmol L^{-1}). Spectrophotometric determinations of PO_4^{3-} were conducted with the same device (LOD = 0.033 µmol L^{-1}). NO_3^- samples were kept in the dark and frozen until spectrophotometric analysis with a Thermo Scientific Evolution™ 201 (Thermo Fisher Scientific, Waltham, USA) (LOD(NO_3^-) = 0.162 µmol L^{-1}). Samples for the determination of chlorophyll *a* (chl *a*) and particulate organic matter (POM) concentrations were taken in
3.8 L pre-washed plastic bottles. Subsamples of each container (1 L for chl \(a\), 2 L for POM) were filtered onto VWR glass microfiber filters (47 mm, particle retention 1.6 \(\mu \text{m}\)). Filters for chl \(a\) were homogenized in 7 mL 90% acetone, and the filter slurry was incubated overnight at 4 °C. Samples were centrifuged before an aliquot of the supernatant was transferred to a glass cuvette. Fluorescence was measured (Trilogy® Laboratory Fluorometer, Turner Designs Inc., San Jose, USA) before and after acidification to 0.003 N HCl with 0.1 N HCl for 90 seconds. Pre-combusted filters with POM were stored in combusted tinfoil and frozen at −20 °C until analysis. Filters were dried for 24 h at 40 °C for transport and again for 24 h at 40 °C before analysis. Filters were analyzed for total carbon (C), nitrogen (N) and organic carbon (C\(_{\text{org}}\)) content in an Eurovector Euro EA 3000 CHN elemental analyzer (EUROVECTOR Srl, Pavia, Italy). Precision (± SD) of analyses was calculated from low soil standard (OAC 187560) measured after every 5 samples (C: ± 0.032%, N: ± 0.004%, C\(_{\text{org}}\): ± 0.046%). Water samples for DOC analysis were filtered through pre-combusted glass microfiber filters (VWR, 25 mm, particle retention 0.7 \(\mu \text{m}\)) into acid-washed 30 mL high-density polyethylene bottles and frozen at −20 °C within 3 h after sampling. For analysis, samples were defrosted, acidified with 33% HCl to reach pH 2, and analyzed in a TOC-VCPH+ ASI-V elemental analyzer (Shimadzu, Kyōto, Japan) (LOD = 8 \(\mu \text{mol} \text{ L}^{-1}\)). Reference material: zero sample (Milli-Q) after each sample and Hansell’s consensus reference material (Hansell Laboratory, University of Miami, USA) (42.5 \(\mu \text{mol L}^{-1}\)) every 10 samples. For the present study, all available data from October 2013 until April 2014 were used.

**Experimental design**

Eight aluminum frames (50 × 50 cm) were haphazardly deployed on sand patches between the reef outcroppings (Figure 1B). The frames were secured to the substrate with weights, keeping a distance of 1.5 to 2.5 m between frames. The structures were randomly subjected to one of two treatments (n = 4): (1) Exclusion of predators and grazers, hereafter referred to as “simulated overfishing” (frame structure surrounded with plastic net with a mesh size of 2 cm to exclude larger fishes and invertebrate grazers such as sea urchins); and (2) Control (uncovered frame structure, allowing predator access while controlling for possible frame effects) (Figure 1C). Initially, being part of a larger project (see Roth et al. 2015), the experimental design comprised a third treatment: Four additional, semi-closed cages (frame with closed sides but open top) were deployed to only exclude large invertebrate grazers such as sea urchins. Fishes were still able to enter the cage structures. The exclusion of larger invertebrate grazers with semiclosed cages had no significant effect on the community composition, inorganic carbon content and other variables on tiles compared to the controls, thereby resembling open plots in all measured response variables (Roth et al. 2015). Therefore, for simplification, only the control and simulated overfishing treatments were considered for this study. The use of wire mesh with 2–3 cm diameter holes has been considered as “standard” for simulating overfishing in coral reefs (e.g. Zaneveld et al. 2016). The chosen diameter generally excludes most fishes > 10 cm total length. Smaller or juvenile fishes and grazers are able to enter the enclosures, but these smaller organisms generally contribute little to overall grazing rates on reefs and have minimal impacts on the benthic communities. In addition, access by smaller fishes reflects patterns seen under intensive fishing, in which larger fish species are preferentially harvested while leaving smaller size classes of fish (e.g. Mumby et al. 2006).

Experimental treatments were analyzed over a 6-month period of which 14 weeks corresponded to the non-upwelling and 10 weeks to the upwelling periods to evaluate the isolated and the combined effects of nutrient availability and simulated overfishing. Overall, this experimental design resulted in four different tested conditions: 1) Exclusion of predators and grazers during the non-upwelling period (simulated overfishing); 2) No exclusion of predators during the upwelling period (natural eutrophication); 3) Exclusion of predators and grazers during the upwelling period (simulated overfishing and natural eutrophication); and 4) No exclusion of predators before upwelling (control/reference).

Each of the frames was equipped with 24 rough, untreated, terracotta tiles. Each tile was about 8.4 × 19 cm and had an average (± SE) surface area of 168.8 ± 0.8 cm\(^2\). Rough terracotta tiles were used, as their surface simulates coral rock and enhances natural species richness and biomass compared to other artificial substrates (Fitzhardinge and Bailey-Brock 1989; Whalan et al. 2015). Settlement tiles were positioned pairwise on top of each other, resulting in 12 light-exposed tiles (mimicking upwards facing, well-lit substrates) and 12 tiles facing downwards (mimicking cryptic, shaded substrates) per frame. To reduce sedimentation, tiles were installed in a 45-degree angle relative to the substrate on a tough plastic net fixed between the vertical poles (Figure 1C). Every two weeks, a random pair of tiles (light-exposed and shaded) was collected from each
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frame, and replaced by a new pair of tiles that were only kept on the reef for the following two weeks. This procedure resulted in two data sets: (1) Long-term succession, where the establishment of organisms on tiles could be observed over the whole 24 week study period with biweekly resolution; (2) Short-term succession, where the establishment of organisms within two-week periods could be observed with changing start dates over the study period. In total, 352 settlement tiles were analyzed for this study: 192 tiles that were initially installed for the long-term observations, and 160 tiles that were installed at intervals of two weeks for the short-term observations.

**Response variables on settlement tiles**

After removing the tiles from the frames, they were immediately photographed under water for later assessment of changes in community composition and relative tile cover. Photos were analyzed with the software Coral Point Count with Excel extension (CPCe) 4.1 (Kohler and Gill 2006) using 100 randomly overlaid points which were then assigned to the following functional groups: (1) Non-biotic cover / bare terracotta; (2) Filamentous algae; (3) Fleshy macroalgae: brown macroalgae; green macroalgae; red macroalgae; (4) Scleractinian corals (5); Sessile calcifying invertebrates other than corals: barnacles; bryozoans; polychaetes; molluscs; (6) Cyanobacteria; (7) Crustose Coralline Algae (CCA); (8) Crustose algae other than CCA; (9) Sponges; and (10) Tunicates. Additionally, the number of *Didemnum* sp. colonies was counted on each short-term succession tile and their maximum linear extension (colony size in mm) measured after two weeks. In the laboratory, all tiles were rinsed with freshwater to remove mobile invertebrates, sediments and salt. All sessile organisms (including algae, invertebrates etc.) were scraped off with razor blades and collected in pre-combusted, pre-weighed, aluminum foil, and dried at 40 °C for 24 h. Samples were homogenized using mortar and pestle for elemental analysis of total carbon (C) and organic carbon (C<sub>org</sub>) content. Ground-powdered samples were weighed to 1 mg and put into 10 × 10 mm silver (C<sub>org</sub>) and tin (C) cups. For analysis of C<sub>org</sub> content, 200 μL 1 N HCl was added to each sample to remove CaCO<sub>3</sub> before being dried again at 40 °C for 24 h. Elemental analysis was carried out with an Eurovector Euro EA 3000 elemental analyzer (EUROVECTOR Srl, Pavia, Italy) with a precision (SD) of ± 0.18% for C (calculated with Apfelblatt SRM 1515 standard). The inorganic carbon content was calculated as the difference between C and C<sub>org</sub>.

**Species identification**

Reoccurring adult colonial ascidians in the reef and on settlement tiles were identified as *Didemnum cf. perlucidum* (Monniot F., 1983) based on visual morphology (e.g. external features of the colony, cloacal channels) (Kott 2001; personal communication R. Rocha – Universidade Federal do Paraná, Brazil – and M. Carman – Woods Hole Oceanographic Institution, USA) their geographic distribution (Carman et al. 2011; Dias et al. 2016), and on their possible occurrence on tropical coral reefs (Muñoz and McDonald 2014). Juveniles and new recruits were too young to be identified based on morphological features, but since their occurrence was in the same experimental plots, it is highly probable that they were also *D. cf. perlucidum*, and they were treated as such for later calculations.

**Statistical analysis**

Statistical analysis was performed using SigmaPlot 12.5 statistic software and PRIMER-e v6 with PERMANOVA+ add-on (Clarke and Gorley 2006). Data were tested for Gaussian distribution with normal probability plots (Q-Q-plot) and / or Shapiro-Wilk-Test prior to analysis. Environmental background data were grouped into non-upwelling (October 2013 – January 2014) and upwelling (February 2014 – April 2014) periods and tested for differences with a One-way Analysis of Variance (ANOVA). Changes in the benthic community structure on settlement tiles after 24 weeks were explored by ordination methods, specifically the Principal Coordinates Analysis (PCO). For this, all tiles (n = 192) of the long-term succession were used. Effects of simulated overfishing and eutrophication on the accumulation of inorganic carbon (n = 196 tiles), *D. cf. perlucidum* colony size (n = 160 tiles) and the number of recruits (n = 160 tiles) were explored using two-way Analysis of Variance (two-way ANOVA) with “predation” (control vs. simulated overfishing) and “nutrient level” (upwelling vs. non-upwelling) as fixed factors. Holm-Sidak Tests were used for post-hoc pairwise comparison to test for interactive effects of simulated overfishing and eutrophication, respectively. If the interactive term *predation × nutrient level* was significant, a Holm-Sidak Test was used for post-hoc comparison against the control group (control and non-upwelling) to check whether the single effects could be isolated. The interaction terms of the two-way ANOVA were also used to confirm if synergistic or additive effects were observed among groups (Slinker 1998). A two-way Permutation Multivariate Analyses of Variance (two-way PERMANOVA) that
Table 1. Environmental variables under non-upwelling (October 2013 – January 2014) and upwelling (February 2014 – April 2014) conditions at Matapalo reef. Average values ± SE were calculated from all available data between 21 October 2013 and 31 March 2014. The number of replicates for each variable and season is displayed in brackets. P-values are derived from the comparison of non-upwelling and upwelling conditions using one-way Analysis of Variance.

<table>
<thead>
<tr>
<th>Water variable</th>
<th>Non-upwelling (Oct – Jan)</th>
<th>Upwelling (Feb – Apr)</th>
<th>ANOVA P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water temperature [°C]</td>
<td>28.09 ± 0.26 (15)</td>
<td>25.49 ± 0.70 (9)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Light [µmol photons m⁻² s⁻¹]</td>
<td>1269 ± 218 (15)</td>
<td>1885 ± 258 (9)</td>
<td>0.089</td>
</tr>
<tr>
<td>Phosphate [µmol L⁻¹]</td>
<td>0.20 ± 0.04 (34)</td>
<td>0.46 ± 0.13 (27)</td>
<td>0.040</td>
</tr>
<tr>
<td>Ammonium [µmol L⁻¹]</td>
<td>0.51 ± 0.05 (41)</td>
<td>1.13 ± 0.22 (27)</td>
<td>0.003</td>
</tr>
<tr>
<td>Nitrate [µmol L⁻¹]</td>
<td>0.30 ± 0.07 (42)</td>
<td>1.87 ± 0.73 (27)</td>
<td>0.014</td>
</tr>
<tr>
<td>Chlorophyll a [µg L⁻¹]</td>
<td>0.56 ± 0.10 (40)</td>
<td>1.10 ± 0.23 (27)</td>
<td>0.023</td>
</tr>
<tr>
<td>Particulate organic nitrogen [µg L⁻¹]</td>
<td>26.39 ± 3.52 (42)</td>
<td>59.63 ± 11.52 (27)</td>
<td>0.004</td>
</tr>
<tr>
<td>Particulate organic carbon [µg L⁻¹]</td>
<td>237.00 ± 35.08 (42)</td>
<td>367.78 ± 68.72 (27)</td>
<td>0.006</td>
</tr>
<tr>
<td>Dissolved organic carbon [µmol L⁻¹]</td>
<td>139.31 ± 14.67 (39)</td>
<td>168.31 ± 14.36 (26)</td>
<td>0.018</td>
</tr>
</tbody>
</table>

Figure 2. Principal Coordinates Analysis (PCO) of shifts in community composition on settlement tiles after 24 weeks. Bi-weekly sampling data from October 2013 to April 2014 were grouped by the factor predation (simulated overfishing and control). Data points reflect community composition on tiles in simulated overfishing (n = 98 tiles) and control (n = 98 tiles) plots. The distance between the points reflects their similarity in community composition (close = similar), and the shift along the axes can be assigned to changes in benthic cover by major functional groups and the ascidian *D. cf. perlucidum*. CCA = Crustose coralline algae.

Results

Environmental conditions

Water temperatures at the studied reef were about 28 °C from October 2013 to January 2014. Temperatures dropped repeatedly by 3–5 °C during upwelling events between February and April 2014 (Table 1). These drops in temperature were accompanied by 2-, 2-, and 6-fold increases in phosphate, ammonium and nitrate concentrations, respectively. Along with the increase in nutrients, chlorophyll *a*, PON, POC and DOC increased significantly by around 100%, 130%, 55% and 20%, respectively. Even during upwelling events, chl *a* levels were relatively low and light availability did not differ significantly between upwelling and non-upwelling observations.

Effects of simulated overfishing

Multivariate analysis revealed pronounced changes in the benthic community composition (Figure 2) that were significant under the influence of simulated overfishing.
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**Figure 3.** Exemplary pictures of benthic communities in the reef (A) and on settlement tiles (B–E). *Didemnum cf. perlucidum* between branches of the coral *Pocillopora* sp. (A). Newly settled *D. cf. perlucidum* colonies on bare settlement tiles (B). After 24 weeks, settlement tiles (control treatment) were inhabited by diverse encrusting organisms (C). The exclusion of predators resulted in a dominance of *D. cf. perlucidum* on shaded (D) and light exposed (E) settlement tiles. Scale bar: 1 cm, applicable for all images A–E. Photographs by F. Roth.

(two-way PERMANOVA – factor predation, Pseudo-$F_{(11,28)} = 42.765$, $P_{(perm)} = 0.001$, perms = 997, n = 196 tiles). The community composition in the simulated overfishing treatment shifted from encrusting organisms (Figure 3C) towards a dominance of the colonial ascidian species *D. cf. perlucidum* (Figure 3D–E). PCO1 explained 66.2% of the total variance in the data and correlated positively to cover of *D. cf. perlucidum* (Pearson correlation, $r = 0.97$) and negatively to CCA ($r^2 = -0.89$) and calcifying invertebrates ($r^2 = -0.74$). The community composition shifted further along PCO2, which explained 21.7% of the total variance, and showed a positive correlation to macroalgae ($r^2 = 0.64$) and *D. cf. perlucidum* ($r^2 = 0.39$) and a negative correlation to non-biotic cover ($r^2 = -0.77$) and filamentous algae ($r^2 = 0.56$). After 24 weeks, *D. cf. perlucidum* was the dominant species with 74–86% relative cover on settlement tiles (further details on the community composition can be found in Roth et al. (2015)).

Simulated overfishing resulted in a 16-fold decrease in the accumulation of inorganic carbon on settlement tiles (two-way ANOVA – factor predation, $df = 1$, $F = 59.388$, $P = < 0.001$, n = 196 tiles). However, two-way ANOVA also showed a significant interaction between the factors *predation* and *nutrient level* (interaction term to be discussed later), indicating that the factors *predation* and *nutrient level* may not be independent. Nevertheless, post-hoc comparisons against the control group indicates an isolated effect by simulated overfishing on the accumulation of inorganic carbon (Holm Sidak test
Figure 4. Accumulation of inorganic carbon on long-term succession settlement tiles. Grey box indicates the period of coastal upwelling. Data points presented as means ± SE (shaded regions) over the study period of 24 weeks. Dashed lines represent a fitted curve that directly connects the data points. The curve fitting involves interpolation and is subject to a degree of uncertainty.

Figure 5. Number of D. cf. perlucidum colonies (A) and their average size (B) after two weeks on short-term succession settlement tiles in the control and simulated overfishing treatment. Grey box indicates the period of coastal upwelling. Data points presented as means ± SE (shaded regions) over the study period of 24 weeks. Dashed lines represent a fitted curve that directly connects the data points. The curve fitting involves interpolation and is subject to a degree of uncertainty.

against the control group control and non-upwelling, P < 0.001, n = 112 tiles) (Figure 4).

Simulated overfishing significantly enhanced the primary succession of D. cf. perlucidum that was assessed through the average number of newly settled colonies per tile (Figure 5A) and their average colony size after two weeks on short-term succession tiles (Figure 5B). Here, the exclusion of predators significantly increased the mean number of D. cf. perlucidum colonies from 2.6 to 9.6 per tile (two-way
ANOVA – factor predation, \( df = 1, F = 274.360, P < 0.001, n = 160 \) tiles and significantly enhanced the average linear colony size from 2.93 to 3.41 cm by 16% (two-way ANOVA – factor predation, \( df = 1, F = 13.529, P = 0.002, n = 160 \) tiles).

**Effects of natural eutrophication caused by seasonal upwelling**

Seasonal changes in water variables did not cause a significant shift in the benthic community succession on long-term settlement tiles (two-way PERMANOVA – factor nutrient level, \( P = 0.172, n = 196 \)). However, analysis of short-term succession tiles revealed a significant alteration in the primary succession of *D. perlucidum*. Coastal upwelling significantly (two-way ANOVA – factor nutrient level, \( df = 1, F = 38.420, P < 0.001, n = 160 \) tiles) enhanced mean *D. cf. perlucidum* colony size after two weeks from 2.79 mm (before upwelling) to 4.17 mm (during upwelling). The increase in size was positively correlated (Pearson correlation, \( r^2 = 0.89 \)) to seawater POM concentrations. The average number of *D. cf. perlucidum* colonies per tile remained constant throughout the study period (Figure 5A) \( (P = 0.707) \).

**Combined effects of simulated overfishing and natural eutrophication**

Two-way ANOVAs revealed that simulated overfishing and eutrophication through seasonal coastal upwelling had interactive effects on the accumulation of inorganic carbon on settlement tiles (two-way ANOVA – factor predation \* nutrient level, \( df = 1, F = 14.891, P < 0.001, n = 160 \) tiles). The combination of the simulated overfishing treatment and eutrophication was negative synergistic and caused a decrease in the accumulation of inorganic carbon during coastal upwelling (Figure 4) (Holm-Sidak comparison of simulated overfishing vs. upwelling, \( Diff \) of means = 2.454, \( t = 6.874, unadjusted \) \( P < 0.001, n = 80 \) tiles). On short-term succession tiles, simulated overfishing and eutrophication showed an additive effect on the early colony size of *D. cf. perlucidum* on short-term succession tiles (two-way ANOVA – factor predation \* nutrient level \( df = 1, F = 2.826, P = 0.110, n = 160 \) tiles) (Holm-Sidak comparison of simulated overfishing vs. upwelling, \( Diff \) of means = 1.861, \( t = 5.233, unadjusted \) \( P < 0.001, n = 80 \) tiles). The number of *D. cf. perlucidum* colonies per settlement tile was not affected by the combined effect of simulated overfishing and natural eutrophication (two-way ANOVA – factor predation \* nutrient level, \( df = 1, F = 0.532, P = 0.475, n = 160 \) tiles).

**Discussion**

Globally, the observed rapid, large-scale loss of coral cover has often been associated with local stressors, including but not restricted to overfishing and eutrophication (Bellwood et al. 2004; Fabricius 2005; Smith et al. 2010). While phase-shifts from coral to algal dominance have been a focus of many studies (e.g. McCook et al. 2001; Mumby 2009; Roff et al. 2015), only a few reports have discussed the role of filter-feeding organisms in changing reef environments (reviewed in Norström et al. 2009). The results of the present study show that the colonial ascidian *D. cf. perlucidum* was the predominant fouling organism in the investigated reef under the combined pressure of simulated overfishing and natural eutrophication, showing a competitive advantage over other benthic taxa, including important reef calcifiers.

**Species identification and status in Central America**

Colonial ascidians in experimental plots and in natural reef habitats were identified based on external morphological features, their geographic distribution, and their possible occurrence on tropical coral reefs. However, many members of the family Didemnidae show similarities in morphological characteristics, including shape and color of the colony, and the size of zooids, larvae, and spicules. Hence, DNA barcoding involving the sequencing of the COI gene is recommended for the identification of ascidians (Jaffarali et al. 2016). Thus, to prevent any misinformation, the open nomenclature *Didemnum cf. perlucidum* is used, indicating that the specimen is believed to be *Didemnum perlucidum*, but the actual identification cannot be certain. Importantly, the study assesses the effects of a non-indigenous organism on ecosystem functioning under predicted environmental change, and is not a record of an ascidian species in a certain region.

The geographic origin of *D. cf. perlucidum* remains unknown. The Center for Research in Marine Science and Limnology (CIMAR) of the University of Costa Rica (UCR) initiated the exploration of the marine environments and organisms in Costa Rica in the early 1990’s. In 2015, the BioMar ACG project was initiated to create an inventory of the species in habitats of Costa Rica, with no study recording *Didemnum perlucidum* in waters of the Pacific coast (e.g. Cortés 2017; Cortés and Wehrtmann 2009). Only recently, *Didemnum perlucidum* has been recorded and classified as invasive to the waters of the Pacific coast of Panama and along the Central American coast (Carman et al. 2011), consequently, it was considered non-indigenous for the present study.
Effects of simulated overfishing

*D. cf. perlucidum* successfully colonized settlement tiles in all treatments and at all times during the study period, a common feature of many invasive ascidian species (Kremer et al. 2010; Lambert and Lambert 2003). However, enhanced recruitment success and an increased average colony size after two weeks were observed under the effects of simulated overfishing, leading to a drastic shift in the community composition contributing to up to > 80% relative cover on the settlement tiles. These observations exceed by far values reported from other studies (ranging from 1 to 35% cover) that assessed relative cover on new settlement substrate under natural conditions (Shenkar et al. 2008) or predation exclusion treatment (Kremer and Rocha 2011). These findings emphasize the importance of native predators in controlling the establishment of invasive species (Kremer and da Rocha 2016). Particularly for susceptible species, such as many ascidians with a soft tunic, predation can be extremely important to impede domination and growth (Kremer and da Rocha 2016). The successful establishment of *D. cf. perlucidum* is likely influenced by the availability of refuges from predators during the post settlement processes. Indeed, in the natural reef environment, most of the colonies of *D. cf. perlucidum* were observed in crevices and at the base of coral branches of *Pocillopora* spp. where predator access is limited (Figure 3A).

In the present study, community dominance by *D. cf. perlucidum* in experimental plots resulted in a widespread loss of encrusting and calcifying organisms, such as CCA, barnacles, serpulids, and molluscs. These shifts in the benthic community composition, from calcifying organisms towards *D. cf. perlucidum*, caused a reduced accumulation of CaCO$_3$. This may result in an imbalance between construction and destruction processes of reef calcification/cementation versus erosion. The consequences may be severe, as inorganic and biogenic CaCO$_3$ bind framework components and occlude porosity in a healthy reef environment (Perry and Hepburn 2008). That means, poorly cemented reef frameworks are only supported by a thin envelope of encrusting organisms and an organic matrix of other infauna that are more susceptible to erosion than a developed community of calcifiers (Manzello et al. 2008). The competitive advantage of *D. cf. perlucidum* over other benthic organisms may partially result from its continuous reproductive potential. *D. cf. perlucidum* not only produces larvae all year round, but colonies also grow and expand by means of vegetative budding and reattachment of fragments (Muñoz et al. 2015). Indeed, we observed persistent recruitment on both control and simulated overfishing settlement tiles throughout the study period (Figure 3B and Figure 5A), suggesting that *D. cf. perlucidum* populations in the reef generate a constant source of propagules, as shown for other tunicates (Dumont et al. 2011; Ruiz et al. 2009).

Effects of natural eutrophication

An effect of natural eutrophication was detected by the increased colony sizes of newly settled *D. cf. perlucidum* colonies after two weeks in both the control and simulated overfishing treatment (Figure 5B) that was attributed to increased levels of POM in the water column (Pearson correlation, $r^2 = 0.89$). Coastal upwelling resulted in higher nutrient concentrations, increasing POM and chlorophyll $a$ content in the water and may have led to a higher availability of food for filter feeding organisms (Rodríguez-Martínez et al. 2012). This finding is concordant with the results of Muñoz et al. (2015), showing positive correlation between *D. cf. perlucidum* growth and chlorophyll $a$ concentrations ($r^2 = 0.958$) in the waters of Western Australia. Eutrophication may enhance the growth of filter feeders, such as ascidians, by providing a nutritional advantage over numerous other reef organisms that are mainly autotroph and / or adapted to more oligotrophic environments, as previously suggested by Shenkar et al. (2008).

Interactive effects of simulated overfishing and natural eutrophication

Simulated overfishing and eutrophication through seasonal coastal upwelling had additive effects on growth rates of *D. cf. perlucidum* and negative synergistic effects on the accumulation of inorganic carbon on settlement tiles. While both a lack of predators and a higher availability of POM are known to enhance *D. cf. perlucidum* growth (Kremer and da Rocha 2016; Muñoz et al. 2015), the negative synergistic effects on the accumulation of CaCO$_3$ had not been explored. A possible explanation for the disproportional relationship may be that autotrophic crustose coralline algae, the main calcifiers on the control settlement tiles, cannot benefit from elevated POM levels and may even be negatively affected by light reduction from turbidity during upwelling events (Fabricius and De’Ath 2001; Fabricius 2005), although significant reduction in light levels was not observed in this study. Although other calcifying suspension-feeders on control settlement tiles, such as barnacles and polychaetes, may also benefit from elevated concentrations of POM, such organisms often have relatively slow
growth rates compared to D. cf. perlucidum (e.g. Sanford and Menge 2001), giving the tunicate a competitive advantage, in the short-term, over native reef organisms. This study suggests that facilitated recruitment – driven by combined effects of (simulated) overfishing and (natural) eutrophication – can increase the competitive success of D. cf. perlucidum over other encrusting species. Almost all native benthic organisms, including functional groups that are favored by a reduced number of fish and an increased availability of inorganic nutrients (e.g. turf- and macro-algae) (Smith et al. 2010), were outcompeted by D. cf. perlucidum. This could negatively affect primary and secondary community succession and ecological functions of the recipient location.

Management implications

The successful establishment of D. cf. perlucidum on a natural coral reef in Eastern Pacific coastal waters of Central America is a newly observed phenomenon and should be carefully monitored. The distribution, biology, and ecological implications of D. cf. perlucidum in Costa Rica are mostly unknown, and demand urgent investigation. Management strategies must include phase shift mitigation that take into account the potential range of alternative states beyond the widely-recognized coral-algal phase shifts. Further research should, therefore, aim at understanding the conditions under which phase shifts to different states, e.g. didemnid ascidian dominance, are likely to occur. Moreover, distribution vectors, such as recreational and fishing vessels that tend to account for most ascidian introductions and spreads (Kauano et al. 2016), need to be monitored and evaluated for their potential to spread D. cf. perlucidum. The consideration of the cumulative impacts of multiple drivers of change will need to become a greater focus for adaptive management strategies.

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