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Community Structure of Periphytic Diatoms in Early-stage Marine Biofilms in a Mariculture-impacted Area

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Periphytic diatoms constitute a major part of complex unicellular aggregations called marine biofilm or periphyton, of which little is known about in tropical marine environments. The early-stage community structure of periphytic diatom genera on two artificial substrates – glass slide and polycarbonate sheets – was characterized. These artificial substrates were placed underwater for 6 d and examined on Days 1, 3, and 6 in four sites around Santiago Island, Bolinao, Pangasinan, Philippines, with different relative distances from the intensive milkfish mariculture area. Correlations of diatom community structures in these sites with physico-chemical factors were investigated. The five most abundant diatom genera – namely, *Cylindrotheca*, *Nitzschia*, *Navicula*, *Amphora*, and *Pleurosigma* – exhibited a weak correlation with nutrients. Less abundant genera *Pseudonitzschia, Haslea*, *Bacillaria*, *Thalassionema*, *Rhizosolenia*, *Eucampia*, *Diploneis*, *Asterionellopsis*, *Chaetoceros*, *Bacteriastrum*, *Licmophora*, *Skeletonema*, *Lioloma*, *Thalassiosira*, and *Thalassiothrix* showed a positive correlation with nutrients. Among sites, the highest benthic diatom cell densities (4.6×10^5 cells cm^{-2}) after 6 d was found in Lucero. Generic richness and diversity varied among sites, with the highest diversity (H' = 1.58) on Day 1 in Tomasa, the site nearest to the mariculture area, whereas the highest richness (D = 2.17–3.26) for all days and diversity (H' = 1.38–1.52) for Days 3 and 6 were recorded in Silaqui, the most distal site. Generally, the highest diversity and richness were found on Day 1, which then decreased on succeeding days in all sites. The lack of a clear pattern in community structure among sites relative to the proximity of these sites to the mariculture area may in part be attributed to the presence of other nutrient sources. Results of the study provide baseline information on the variability of periphytic diatom community structure in a mariculture-impacted area, and insights on how benthic diatoms may be used in monitoring the impacts of nutrient pollution.

Keywords: benthic diatom, community structure, mariculture, marine biofilm

INTRODUCTION

Marine biofilms, also known as periphyton, are complex biological aggregations of unicellular organisms composed predominantly of marine bacteria, Archaea, benthic microalgae, and flagellates attached to submerged substrates (Dobretsov 2010). These biological complexes influence the structure of benthic communities by inducing or inhibiting the settlement of algal spores (Patel *et al*. 2003) and invertebrate larvae (Qian *et al*. 2007). They also contribute to intertidal primary productivity, with 18−56% and 19−44% of net primary production in temperate and tropical seagrass ecosystems, respectively. Moreover, they are consumed by various grazing invertebrates and, thus, play a vital role in marine food webs (Klumpp *et al*. 1992).

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The process of marine biofilm development on submerged substrates is commonly referred to as biofouling. The colonization process takes place as soon as the substrate is submerged underwater and involves different processes that may either happen sequentially, overlap, or occur in parallel (Dobretsov 2010). It has been reported that during the early phases of colonization, the community structure of biofilm is influenced by: [1] the presence of microfouling organisms, either in the water column or nearby substrates; and [2] physical and chemical properties of the substrate and the surrounding environment (Chiu *et al*. 2008; Witt *et al*. 2011). Over the past decades, many man-made structures have been built in marine coastal areas. Significant differences in cell densities and diversity have previously been found between benthic diatom communities in artificial substrates like fiberglass and glass coupons (Patil and Anil 2005b).

Nutrient availability is one environmental factor that influences the development of marine biofilm communities. Previous studies by Agatz *et al*. (1999), Mitbavkar and Anil (2007), and Kafouris *et al*. (2019) showed that the community structure of benthic diatoms was affected by nitrogen and phosphorus enrichment. Increased nutrient loading in the mariculture-impacted waters of Bolinao, Pangasinan, Philippines affected the community structure of planktonic diatoms and dinoflagellates (Yap *et al*. 2004; Azanza *et al*. 2005, 2006; San Diego-McGlone *et al*. 2008). Yap *et al*. (2004) found differences in the relative abundance of planktonic diatoms and dinoflagellates across sites with varying impacts from the mariculture area. Whether this is true for benthic diatoms has not been investigated. Unlike in freshwater systems, there is no information on the effect of elevated nutrient loading on periphytic diatoms, especially in mariculture-impacted areas (Desrosiers *et al*. 2013). To address this knowledge gap, this study characterized the diatom component of biofilm communities during the early stages of their establishment and succession in coastal areas with nutrient influence from mariculture activities (Villanueva *et al.* 2006; Tanaka *et al.* 2014; Ferrera *et al.* 2016). The study analyzed differences in the community structure of benthic diatoms, based on their composition and relative abundance, in four sites with varying distances from the intensive mariculture area, as well as the correlation of abundances of different benthic diatom genera to respective water parameters. Two types of artificial substrates – glass slides and polycarbonate sheets – were used simultaneously in this study to evaluate to what extent the type of artificial substrate affected the benthic diatom community structure in the sampling sites. This study provides baseline information on the early successional phase of periphytic microalgal communities and insights into its potential use for the monitoring of nutrient pollution in the marine environment.

MATERIALS AND METHODS

Site Description

The study was conducted in sites within a mariculture-impacted coastal area around Santiago Island in Bolinao, Pangasinan in northwestern Philippines (N 16°23'19", E 119°53'34"). Due to intensive milkfish (*Chanos chanos* Forsskal) mariculture activities, the coastal water quality in the area has deteriorated (Ferrera *et al*. 2016). Local wind- and tidal-driven current patterns facilitate the transport of nutrients from the mariculture area to adjacent sites (Rivera 1997), which may lead to differences in nutrient levels of these sites relative to mariculture proximity. Four (4) sites were chosen in this study – namely, Tomasa, Lucero, Silaqui on the western side of Santiago Island; and Victory on the eastern side (Figure 1). Tomasa (N 16°23'7.42", E 119°54'38"), located at the mouth of Guiguiwanen Channel adjacent to the mariculture area (~ 500 m from the nearest fish cage), is characterized by having silt and soft sediment and the presence of macroalgae and few corals. Lucero (N 16°24'43", E 119°54'14"), located ~ 2.5 km north of Tomasa, has both coral and seagrass habitats. Silaqui (N 16°26'24", E 119°55'30") is a seagrass-dominated area located ~ 3.9 km northeast of Lucero and is farthest from the mariculture area. Victory (N 16°23'35", E 119°58'1.2") is a seagrass-dominated marine protected area located on the eastern side of Santiago Island opposite Tomasa and is ~ 3 km away from the mariculture area. Tomasa has a water depth of ~ 4 m, whereas all the other sites are ~ 1.5 m deep. These sites have, therefore, varying proximity to the mariculture area, with Victory and Lucero having comparable proximity.

Sampling of Water Parameters

Water samples were collected only once *via* point sampling at the start (Day 0) of the 6-d experiment using a 5-L Niskin water sampler (General Oceanics, Inc.) at 1 m below the surface. On-site measurements of temperature, salinity, and turbidity were done using a multi-parameter water quality meter AAQ-RINKO (JFE Advantech Co., Ltd.). For dissolved oxygen (DO), water samples were analyzed using the Winkler method. Samples for measurement of carbonate concentrations [total alkalinity (TA), dissolved inorganic carbon (DIC), and pH] and water quality parameters (nutrients, DO, chlorophyll-a, and total suspended solids (TSS)] were also collected. Samples for TA and pH were analyzed using the ATT-05 Total Alkalinity Analyzer (Kimoto). Both these parameters are needed to calculate in situ pH using the CO2SYS software (van Heuven *et al.* 2011). For nutrients (nitrite, nitrate, ammonium, phosphate, and silicate), samples were analyzed using a UV-Vis Spectrophotometer (Shimadzu Mini 1620), following the method of Parsons *et al*. (1984).

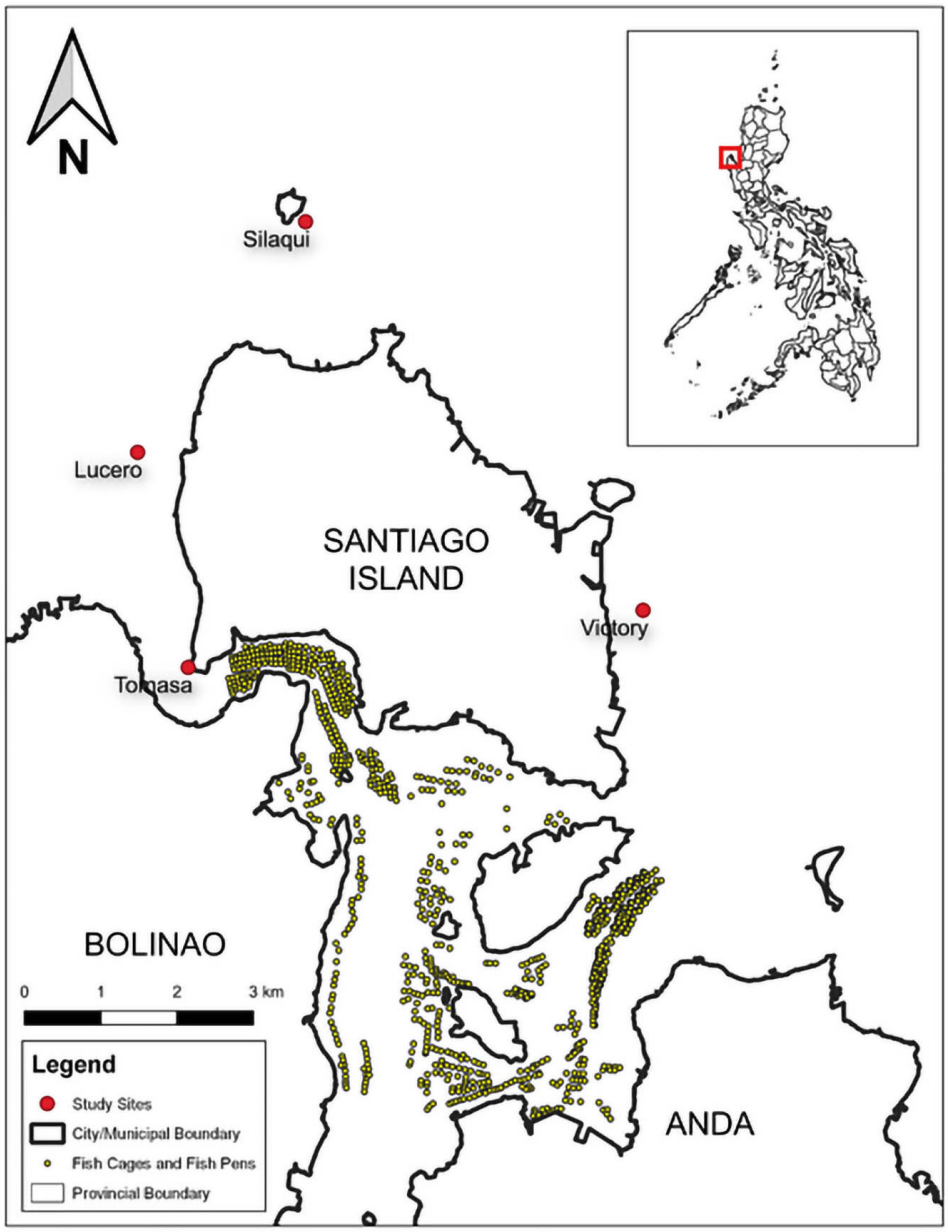

Figure 1. A map of the Bolinao-Anda reef complex showing the four study sites and mariculture area. The inset map shows the location of this reef complex within the Philippines. Map generated by T.J. Cipriano.

Water samples (500 mL) for TSS were filtered using pre-weighed glass-fiber filters and were dried to constant weight at 100 °C.

Sampling of Periphytic Benthic Diatoms

Acid-washed glass slides and polycarbonate sheets (2.5 cm × 7.6 cm) were hung in racks near the bottom (~ 10 cm) in the four sites for 6 d from 27 Apr–02 May 2018. In each site, three racks containing 15 glass slides each and another set of three racks of 15 polycarbonate sheets each were deployed for a total of 45 glass slides and 45 polycarbonate sheets per site. Each rack was placed approximately 5 m apart from each other. Three (3) replicates from each of the three racks per substrate from each site were retrieved after 1, 3 and 6 d of deployment for a total of nine replicates per substrate type retrieved from each site per sampling day. Samples were then brought to the laboratory for microscopy. Sampling was terminated on the 6th day due to the appearance of invertebrate grazers on the substrates observed in Tomasa.

Substrates were pre-rinsed with sterilized seawater to dislodge loosely attached objects, after which diatom

samples were scraped from the substrates using a nylon-bristled brush and placed in 10-mL conical centrifuge tubes (Patil and Anil 2005a). A 1-mL aliquot from each sample was placed in a Sedgewick Rafter counting chamber and observed under a light microscope. For each sample, three replicate counts were conducted (Johnson *et al*. 2015). Diatoms were identified to the genus level using taxonomic guides based on Tomas (1997) and DiatomBase (Kociolek *et al*. 2020). For genera with high cell densities, diatoms from randomly selected grid columns were counted until approximately a 100–200 cell count was achieved (Johnson *et al*. 2015). Calculations were then done using the formula of Johnson *et al*. (2015):

$$\frac{\text{diatom cells}}{\text{mL}} = \frac{\text{diatoms counted x 1000}}{\text{no. of observed grids}} \quad (1)$$

$$\text{diatom cells density} \left(\frac{\text{cells}}{\text{cm}^2}\right) = \frac{\frac{\text{diatoms}}{\text{mL}} \text{ x storage volume}}{\text{surface area substrate}} \quad (2)$$

Cell density was expressed as cells per given area (cells cm^{-2}) and values for different diatom genera were then used to calculate the Shannon diversity index [H' (ln)] and Margalef's richness index (D).

Using data on their relative abundance and presence/absence, benthic diatoms were classified based on dominance and residency using the criteria of Yap-Dejeto and Baqueros (2015). Genera with > 20% relative abundance were considered "dominant," those with 10–20% relative abundance were "secondary," and those with < 10% relative abundance were "accompanying." Genera that were present for at least two consecutive days, either from Days 1–6 or Days 3–6, were classified as "residents," whereas those present only once or twice but not consecutively (*i.e.* present on Day 1, lost on Day 3, and reappeared on Day 6) were considered "transient."

Data Processing and Analysis

The effect of substrate was tested *a priori* by comparing mean cell densities, richness, and diversity between glass and polycarbonate (n = 3) per site at different time points using student's t-test. As substrate has no effect on richness and diversity indices, data for glass and polycarbonate were subsequently pooled for further analyses. However, significant differences in total cell densities between the two substrates were found; thus, data were not pooled. Data were tested for assumptions of normality and variance using Shapiro-Wilk test and Levene's test, respectively. Data that did not conform to normality and equal variances were transformed until assumptions were met. One-way ANOVA was conducted to evaluate differences in mean cell densities per substrate among sites and separately among different time points. If significant differences were found, pairwise comparisons were subsequently done using Tukey's test to find significant groupings among data sets.

Differences in mean richness and diversity among sites and time points were compared using two-way ANOVA. Multiple pairwise comparisons were done afterward using the emmeans package in the R program. All analyses were conducted using R software (R Core Team 2020) with the significance level set at $p = 0.05$.

Bray-Curtis (BC) similarity measure (Bray and Curtis 1957) was calculated based on the presence-absence and cell densities of each benthic diatom genus. Since the data set contains zero counts, values were Hellinger-transformed and clustered based on substrate type, site, and time. Visualization of clustering by ordination was done using principal coordinate analysis (PCoA; Gower 1966). Patterns generated from PCoA were tested using analysis of similarity (ANOSIM; Clarke 1993), which utilizes BC dissimilarity measure within and between sites and sampling days. ANOSIM values at R > 0.5 indicate strong differences, R = 0.25 to < 0.50 indicate differences with some similarities or overlap, R = 0.10 to < 0.25 are similar with some difference or high overlap, and R < 0.1 are highly similar (Clarke 1993). Similarity percentage analysis (SIMPER) was further conducted to determine the percentage contribution of each genus to dissimilarities. Canonical correspondence analysis (CCA) was also done to identify correlations between cell densities of different benthic diatom genera and environmental factors (Ter Braak 1986). Statistical analyses were performed using PAST 4.03 (Hammer *et al*. 2001).

RESULTS

Physico-chemical Characteristics of Sites

The physico-chemical characteristics of the study sites show nutrient differences and trends relative to the site's proximity to the mariculture area (Table 1). Tomasa, the site adjacent to the mariculture area, had the highest phosphate and DIC among all the sites. Meanwhile, Lucero had the highest nitrite and ammonium. Notably, the highest nitrate and lowest nitrite and phosphate were observed in Silaqui, the site farthest from the mariculture area. Along the western side of Santiago Island, phosphate decreased and nitrate increased with distance from the mariculture area. Victory on the eastern side had the lowest nitrate and ammonium, as well as lower nitrite and phosphate, compared to Tomasa and Lucero. The highest silicate was also recorded in Victory. Despite the low phosphate and nitrogen compounds in Victory compared

Table 1. Summary of the physico-chemical parameters at different sites in a mariculture-impacted area in Bolinao, Pangasinan, Philippines.

Site	pH	Temp (°C)	Salinity (ppt)	DO (mg/L)	[NO_3] (µM)	[NO_2] (µM)	[NH_4] (µM)	[PO_4] (µM)	TIN:TIP	[SiO_3] (µM)	TA (µmol/kg)	DIC (µmol/kg)	Turbidity (FTU)	TSS (g/L)
Tomasa	7.96	31.39	34.28	4.81	0.92	0.11	6.15	1.83	3.92	4.39	2,245	2,023	0.40	0.006
Lucero	8.00	30.71	34.10	4.58	1.03	0.12	6.42	0.88	8.60	5.94	2,220	2,010	0.35	0.007
Silaqui	8.21	30.62	33.88	6.73	1.87	0.02	6.37	0.35	23.6	7.63	2,206	1,874	0.46	0.009
Victory	8.55	32.66	34.48	11.0	0.30	0.04	3.10	0.61	5.64	16.33	2,135	1,557	0.48	0.059

to other sites, the TIN:TIP ratio in Victory lies between the ratios in Tomasa and Lucero.

DO was highest in Victory and Silaqui and lowest in Tomasa and Lucero. The highest turbidity and TSS were recorded in Victory, with Tomasa having the lowest pH among sites.

Characteristics of Periphytic Diatom Communities

A total of 21 diatom genera were identified in this study. Fourteen (14) genera belong to eight families of pennate diatoms, whereas the remaining seven genera are from six families of centric diatoms (Appendix Table I). Pennate diatoms were the dominant group, with relative abundance ranging from 77.8–99.7% across all samples. Seven (7) genera were found in all sites (Appendix Figure I): *Nitzschia*, *Cylindrotheca*, *Navicula*, *Amphora*, *Pleurosigma*, *Thalassiothrix* (pennates), and *Coscinodiscus* (centric). Except for *Thalassiothrix*, all of these genera occurred on all sampling days (Appendix Table I). *Cylindrotheca* and *Nitzschia* appeared to be the dominant genera in all sites with relative abundances of 14.3–69.6% and 18.5–44.3%, respectively (Figure 2). Other genera had a brief period of dominance, which include *Navicula* in Tomasa on Day 1 (glass, 22.3%; polycarbonate, 26.2%), and *Chaetoceros* in Silaqui on Day 6 (polycarbonate, 20.2%). *Navicula* also appeared to be a secondary taxon in Tomasa and Lucero on Day 6 (glass, 11.9–12.3%; polycarbonate, 12.1%) and Victory on Day 3 (polycarbonate, 10.8%). *Amphora* was primarily a secondary taxon in Tomasa (13.9–17.2%), Silaqui (10.0%), and Victory (12.2–16.5%). The remaining ones were all accompanying genera (< 10%).

Resident genera for all sites include the most abundant diatoms – namely, *Nitzschia*, *Cylindrotheca*, *Navicula*, *Amphora*, *Pleurosigma*, and *Coscinodiscus*. Other residents include *Licmophora* in Tomasa and Silaqui, *Chaetoceros* in Silaqui, and *Lioloma* in Victory (Appendix Table I). Most resident genera were pennate diatoms, except for *Coscinodiscus* and *Chaetoceros* (centric). The remaining genera were primarily present in one or two sites, or only sampled on Day 1 and, thus, were considered transient. Only five genera were found uniquely in a single site. Of particular interest was the presence and higher abundance of *Chaetoceros* in Silaqui on Days 3 and 6, even though it is generally planktonic (Yap-Dejeto and Baqueros 2015). Other unique genera include *Rhizosolenia* in Tomasa, *Pseudonitzschia* and *Eucampia* in Lucero, and *Bacteriastrum* in Silaqui. No unique genus was found in Victory.

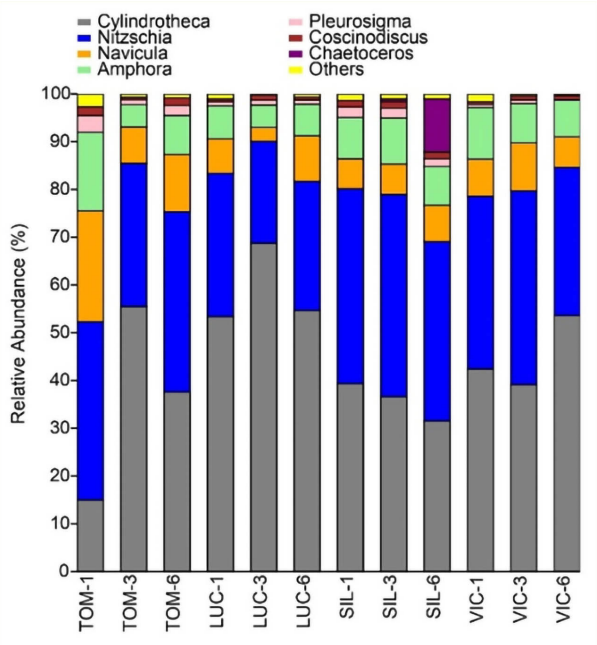

Figure 2. Relative abundance of benthic diatom genera on artificial substrates (pooled data from glass and polycarbonate) at different sites (TOM – Tomasa; LUC – Lucero; SIL – Silaqui; VIC – Victory) on Days 1, 3, and 6. Complete list of diatom genera in Appendix Table I.

Out of the 21 benthic diatom genera identified, four genera were uniquely found on glass – namely, *Bacillaria*, *Pseudonitzschia* (Bacillariaceae), *Asterionellopsis* (Fragilariaceae), and *Eucampia* (Hemiaulaceae). In contrast, there were no unique genera on polycarbonate. Some genera also did not co-occur on glass and polycarbonate in some sites – for example, *Thalassionema*, *Thalassiothrix*, *Lioloma*, *Licmophora*, *Skeletonema*, *Thalassiosira*, and *Eucampia* (Appendix Table I).

Comparing diatom assemblages between glass and polycarbonate, there was no clear pattern as to how the two differed in terms of cell density, richness, and diversity among sites or sampling days (Appendix Table II). Interestingly, diversity between glass and polycarbonate was not significantly different in all sites on Days 1 and 3 but not on Day 6. A significant difference in mean cell density was found among sites in either glass or polycarbonate on all sampling dates (ANOVA, n = 4, $p < 0.05$) (Appendix Table III). Likewise, mean cell density was significantly different among sampling time points in each site in each substrate (ANOVA, n = 3, $p < 0.05$) except for glass in Victory. Overall, Lucero had the highest cell density for both glass and polycarbonate on Day 1, followed by Tomasa, Victory, and Silaqui for both substrates (Appendix Table III). Lucero also had the highest cell density on glass on Days 3 and 6. For polycarbonate, the highest cell density was found in Victory on Day 3 and in Tomasa on Day 6. Diatom assemblages in Silaqui had consistently the lowest cell densities for both substrates on all sampling days. The variable patterns among sites and sampling days indicate that substrate type may not have significant effect on mean cell density and community structure (*i.e.* richness and diversity). This is further supported by results from PCoA (Appendix Figure II), which reveal that community structures between glass and polycarbonate were highly similar (ANOSIM R = –0.047, $p = 0.786$).

Further analysis of richness and diversity was based on pooled data from the two substrates. Two-way ANOVA showed significant interactions between site and time for richness ($p = 0.004$) and diversity ($p = 6.6 \times 10^{-10}$). Significant main effects of site and time were also observed for both parameters. Among sites, Tomasa had the highest diversity on Day 1, whereas Silaqui had the highest richness on all days, as well as the highest diversity on Days 3 and 6 (Table 2). The high diversity and richness in Silaqui and Tomasa could be attributed to the presence of more unique species (Appendix Figure I) that showed higher relative abundance (Figure 2). In contrast, Lucero had the lowest richness and diversity indices during all sampling days, whereas Victory had intermediate values for richness and diversity indices. Diversity and richness in Tomasa, Lucero, and Victory declined from Day s1–6. In contrast, diversity increased but richness decreased in Silaqui from Days 1–6. The increase in diversity in Silaqui coincided with the presence and dominance of *Chaetoceros*, which was not present in other sites on Days 3 and 6.

Community structure based on composition and cell densities of different genera among sites over a 6-d period was highly overlapping (Appendix Figure III; ANOSIM R = 0.139, $p = 0.052$) despite differences in diversity and richness. Comparing between sites, the community structure in Tomasa had strong similarities with that in Lucero (R = –0.113) and Silaqui (R = 0.081) but with high overlap with Victory (R = 0.222). Lucero communities also highly overlapped with Silaqui (R = 0.219) and Victory (R = 0.194). Lastly, communities in Silaqui and Victory were more dissimilar (R = 0.263).

Among diatom genera, *Cylindrotheca* had the highest contribution of up to 12.94% to dissimilarities in benthic diatom communities (Appendix Table IV). Other benthic diatoms that highly contributed to dissimilarities include *Amphora* (11.19%), *Pleurosigma (*10.58%), *Nitzschia* (9.52%), and *Navicula* (9.41%). Among diatom families, Bacillariaceae (*Nitzschia*, *Cylindrotheca*, *Bacillaria*, and *Pseudonitzschia*) had the highest contribution of a total of 23.62% to dissimilarities. The aforementioned genera, while contributing most to dissimilarities, were also dominant within the communities, indicating that changes in less dominant genera among different sites did not contribute to significant changes in the overall community structure between sites.

Early Colonizers and Succession

There were differences with some overlaps in communities among different time points in different sites (Figure 3; ANOSIM R = 0.5097, $p = 0.0001$). The community structure of benthic diatoms on Day 1 was different from those on Days 3 and 6. Early colonizers that were uniquely

Table 2. Comparison of differences in mean (± SD) Shannon diversity (H') and Margalef's richness (D) indices of benthic diatom communities among different sites at different time points in Bolinao, Pangasinan, Philippines using two-way ANOVA.

Site*	Diversity**			Richness**		
	Day 1	Day 3	Day 6	Day 1	Day 3	Day 6
Tomasa	1.58 ± 0.08^{a1}	1.14 ± 0.04^{c3}	1.36 ± 0.07^{ab2}	2.96 ± 0.28^{b1}	1.96 ± 0.04^{ab2}	1.59 ± 0.02^{c3}
Lucero	1.19 ± 0.03^{c1}	0.94 ± 0.02^{d2}	1.12 ± 0.13^{c12}	2.57 ± 0.14^{c1}	1.72 ± 0.11^{b2}	1.58 ± 0.04^{c2}
Silaqui	1.30 ± 0.05^{b2}	1.38 ± 0.09^{a2}	1.52 ± 0.07^{a1}	3.26 ± 0.13^{a1}	2.17 ± 0.18^{a3}	2.40 ± 0.12^{a2}
Victory	1.29 ± 0.07^{b1}	1.24 ± 0.07^{b1}	1.19 ± 0.13^{bc1}	2.94 ± 0.07^{b1}	1.95 ± 0.44^{ab2}	1.86 ± 0.14^{b2}

*Similar letters in superscript denote significant groupings among sites within each sampling day based on multiple pairwise comparisons ($p < 0.05$).
**Similar numbers in superscript denote significant groupings among sampling days within each sampling site based on multiple pairwise comparisons ($p < 0.05$).

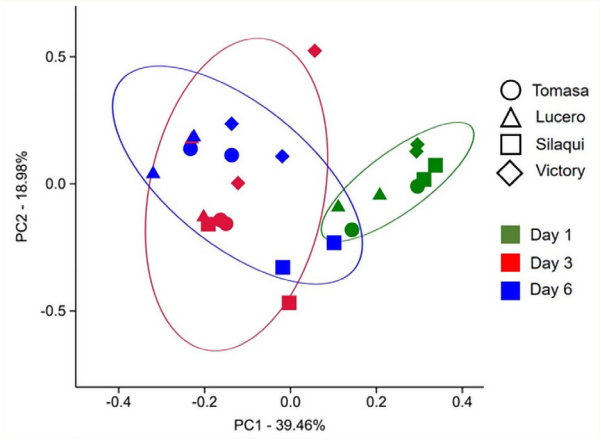

Figure 3. Principal Coordinate Analysis (PCoA; ANOSIM R = 0.5097, p = 0.0001) showing significant separation of the community structure of benthic diatoms recorded between Day 1 and Days 3 and 6 on different substrates in different sites. Each point represents average Hellinger-transformed data of mean total cell counts (density) of all diatom genera on each substrate type within a site on a given day of sampling.

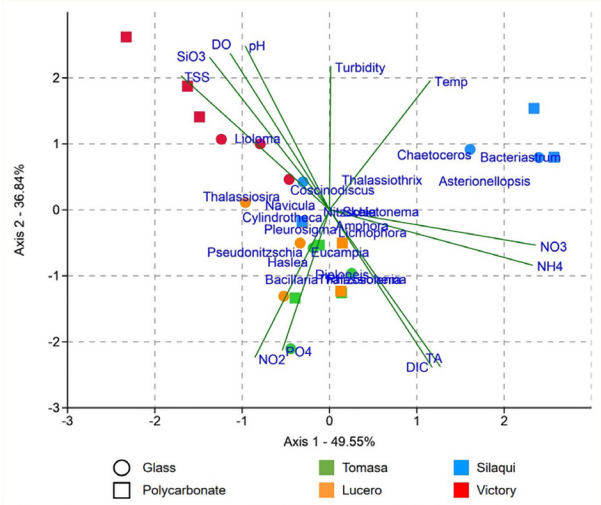

Figure 4. Ordination plot of the canonical correspondence analysis (CCA) showing the distribution of the different benthic diatom genera in different sites (data points per substrate type were separated) in relation to different environmental factors (dark green lines).

found on Day 1 include *Bacillaria*, *Pseudonitzschia*, *Rhizosolenia*, and *Thalassionema*, whereas those that were found on Days 3 and 6 but not on Day 1 include *Eucampia* and *Chaetoceros* (Appendix Table I). The displacement of these four genera found only on Day 1, as well as the increase in the relative abundance of genera such as *Cylindrotheca* and *Chaetoceros* (Figure 2), could have contributed to this clustering. *Cylindrotheca* had the highest contribution of up to 13.40% to dissimilarities in benthic diatom assemblages between Day 1 and Days 3–6 (Appendix Table V). An increasing trend in the relative abundance of *Cylindrotheca* from Days 1–3 was observed particularly in Tomasa. The absence of *Chaetoceros* on Day 1, and its high relative abundance on Day 6 in Silaqui could have also contributed to clustering between Day 1 and Days 3–6.

Relationship between Benthic Diatom Genera and Physico-chemical Parameters

CCA (Figure 4, p = 0.01) showed that differences in the cell densities of different diatom genera in benthic diatom communities among sites were correlated to differences in physico-chemical parameters. Communities in Victory were positively correlated with pH, TSS, turbidity, and DO, whereas those in Tomasa and Lucero were positively correlated with TA and DIC. In relation to nutrients, benthic diatom communities in Tomasa and Lucero showed a positive correlation with nitrite, ammonium, and phosphate; those in Victory exhibited a positive correlation with silicate, and those in Silaqui were positively correlated with nitrate and ammonium.

The six most abundant benthic diatoms (*Cylindrotheca*, *Nitzschia*, *Navicula*, *Amphora*, *Pleurosigma*, and *Coscinodiscus*) showed a weak correlation to all physico-chemical parameters, including nutrients. In contrast, the less abundant genera responded differently to water parameters. *Asterionellopsis*, *Chaetoceros*, *Bacteriastrum*, *Licmophora*, and *Skeletonema* had a positive correlation with DIC and a negative correlation with TA, turbidity, and TSS, whereas *Lioloma* and *Thalassiosira* showed otherwise. *Pseudonitzschia*, *Haslea*, *Bacillaria*, *Thalassionema*, *Rhizosolenia*, *Eucampia*, and *Diploneis* showed a positive correlation with TA and DIC, as well as a negative correlation with turbidity and TSS, whereas *Thalassiothrix* exhibited the opposite trend for TA, DIC, and TSS.

Likewise, the genera that were mostly found in very small numbers and were present only in the communities for a short period of time – such as *Asterionellopsis*, *Chaetoceros*, *Bacteriastrum*, *Licmophora*, and *Skeletonema* – showed a positive correlation with nitrate and ammonia but negative correlation with nitrite, phosphate, and silicate. *Lioloma* and *Thalassiosira* showed otherwise. *Pseudonitzschia*, *Haslea*, *Bacillaria*, *Thalassionema*, *Rhizosolenia*, *Eucampia*, and *Diploneis* showed a positive correlation with nitrate, nitrite, phosphate, and ammonia but negative correlation with silicate, whereas *Thalassiothrix* exhibited the opposite trend.

DISCUSSION

To our knowledge, this study is the first in the Philippines to characterize the generic assemblage of periphytic diatoms in sites impacted by nutrient pollution associated with intensive mariculture activities. Spatio-temporal variations in the benthic diatom community structure were observed. Some correlation was seen between the diatom communities during the early successional phase of biofilm development on the artificial substrates and nutrients with distance from the mariculture area.

Composition and Early Succession Patterns

The most abundant genera in early-stage benthic diatom assemblages based on the frequency of occurrence and cell densities were mostly pennate forms, with the exception of *Coscinodiscus*. This finding corroborates earlier research on marine biofilms which found pennate forms to predominate in benthic diatom assemblages on a variety of natural and artificial substrates (Agatz *et al*. 1999; Patil and Anil 2005a, b; Mitbavkar and Anil 2007, 2008). The dominance of pennate relative to centric diatoms on benthic substrates is clearly expected. Pennate diatoms have an advantage in colonizing substrates due to their raphe, which facilitates their attachment and movement on surfaces (Mitbavkar and Anil 2008; Molino and Wetherbee 2008). This may explain the generally very low relative abundance of most centric diatoms, apart from *Coscinodiscus* and *Chaetoceros*.

In the early stages of biofilm formation, clean surfaces become available for colonization by a plethora of prokaryotic and eukaryotic microorganisms (Dobretsov 2010). In this study, temporal clustering of benthic diatom community structure with some overlap among days was observed, with Day 1 significantly differing from Days 3 and 6. This could be brought about by the general decrease in richness and diversity among sites through time, which could be attributed to the displacement of several genera that are transient in nature, such as several early colonizers (*e.g. Bacillaria*, *Pseudonitzschia*, *Rhizosolenia*, and *Thalassionema*). Transient genera may have attached to surfaces by chance or displaced through competition on subsequent days. The increased dominance of some genera (*e.g. Nitzschia* and *Cylindrotheca*) that are opportunistic to nutrient enrichment could have also resulted in the displacement of other genera (Mitbavkar and Anil 2007). In earlier studies, early-stage biofilms also exhibited temporal clustering such that assemblages at 2–4 d of exposure were different from older biofilms having 6–20 d of exposure (Jones *et al*. 2007).

Benthic diatom communities between glass and polycarbonate were not different in terms of overall community structure. Diversity in the early stages of development (*i.e.* Days 1 and 3) was also not different between the two substrates. The similarity in the diversity and overall community structure between glass and polycarbonate may have been influenced by the formation of a conditioning film in the substrate surfaces. Since the two substrates were deployed under the same environment, the organic matter, which formed on their surfaces would be more or less the same. Such conditioning film could mask the surface chemistry of substrates and, as a result, colonizing species would perceive these different substrates as similar (Jones *et al*. 2007). The formation of such conditioning film, termed molecular fouling, is the first stage in the biofouling of newly-submerged clean surfaces (Dobretsov 2010).

Differences in Community Structure among Sites

Water parameters determined in this study indicate different levels of pH and nutrients with respect to distance from the mariculture area as previously observed by Lagumen and San Diego-McGlone (2014), and Albelda *et al*. (2019). The lowest pH was observed in Tomasa adjacent to the mariculture area, and pH increased with distance. The lower pH in Tomasa is primarily due to high organic loading from unconsumed feeds and fish waste materials in the mariculture area, which – when decomposed – release nutrients and carbon dioxide (Lagumen and San Diego-McGlone 2014; Ferrera *et al.* 2016). The highest phosphate was found in Tomasa closest to the mariculture area, and phosphate decreased in farther sites. This is expected as the excessive use of fish feeds in the mariculture area is one major source of phosphate in the coastal waters of Bolinao (Sumagaysay-Chavoso 2003; Ferrera *et al*. 2016). Unlike phosphate, nitrate increased from Tomasa to Silaqui, whereas Victory recorded the lowest nitrate. Tomasa and Lucero had the highest nitrite, with the values being three to six times higher than those of Silaqui and Victory. Ammonium levels in Tomasa, Lucero, and Silaqui were comparable, with Lucero having the highest. Ammonium levels in these three sites were twice as high as that in Victory. The difference in the levels of nitrogen compounds across sites, with no clear gradient relative to proximity to the mariculture area, indicates the presence of other possible sources of these nutrients. According to Senal *et al.* (2011), elevated levels of nitrogen compounds around Silaqui and Santiago Islands could be from submarine groundwater discharge (SGD) that may have been contaminated with anthropogenic wastes from these islands. Geological features, such as faults, may facilitate the flow of SGDs from Silaqui and Santiago Islands to the reef area (Cardenas *et al.* 2010). The presence of other sources of nutrients, other than the mariculture area, may complicate the behavior of nutrients at the different sites studied.

Based on the TIN:TIP ratio, it was observed that on the western side of Santiago Island, the lowest ratio was in Tomasa and the highest in Silaqui. This suggests that Tomasa is the most N-limited among the sites, which is similar to the findings of Ferrera *et al*. (2016). In contrast, Silaqui is P-limited. On the eastern side, Victory exhibited TIN:TIP ratios characteristic of transition waters, which is comparable to those of Lucero and other sites described by Fortes *et al*. (2012). Similar ratios were reported in Albelda *et al*. (2019) for Tomasa with TIN:TIP < 4, whereas Lucero and Victory are considered transition waters with TIN:TIP = 4–11. Silaqui, which is farthest from the mariculture area, had TIN:TIP > 11. Lucero and Victory lie at comparable distances from the mariculture area, but their nutrient conditions still varied. The discharge from the mariculture area is more evident in the western opening of the channel, where there is also a greater concentration of fish cages compared to the eastern opening (Figure 1). This difference is further influenced by variations in the local wind- and tidal-driven current patterns on different sides of the island that could affect the transport of nutrients from the mariculture area (Rivera 1997; Yoshikai *et al*. 2021). Unlike previous studies where changes in the community structure of corals [*e.g.* Villanueva *et al.* (2005, 2006)], seagrass (Tanaka *et al.* 2014), and seagrass-associated fish (Watai *et al.* 2014) were observed in relation to nutrient gradients along the coast of Santiago Island (Ferrera *et al.* 2016), no such consistent pattern in community structure of the early successional stage of periphytic diatoms was observed in this present study. These periphytic diatoms may be more sensitive to nutrient variability at the microcosmic levels, which may not always reflect the levels detected at any particular site at the macroscopic scale.

The highest cell densities were observed in Lucero, followed by Tomasa. Among sites, Lucero had the highest levels of ammonium and the second-highest level of nitrate that coincided with the highest cell densities relative to other sites. Nitrate and ammonium levels in Tomasa were comparable to that in Lucero, but water depth may have influenced light availability for benthic diatom growth in the site since Tomasa is deeper (4 m) than Lucero (1.5 m). Silaqui recorded the highest nitrate and second-highest ammonium, yet this did not translate to higher cell densities. The low cell densities in Silaqui and Victory suggest that there could be other limiting factors. One potential factor is the placement of substrates within the site. Substrates in Silaqui and Victory were deployed in seagrass areas with thick vegetation, unlike in Tomasa and Lucero where the coral cover and seagrass vegetation were sparse. The thick seagrass canopy may have reduced light availability for diatom growth. Additionally, high TSS and turbidity in Silaqui and Victory may have further reduced light availability. Overall, total cell density may not be a good indicator for monitoring the impact of nutrient pollution because the population growth of different genera in the periphyton assemblage may respond differently to different nutrients and environmental factors.

Despite the difference in physico-chemical parameters among sites, only community structures based on diatom composition and relative abundance in Silaqui and Victory were distinct from each other, whereas the rest were similar or overlapped with each other. The weak correlation of the more dominant genera to nutrients suggests that the population growth of these genera did not respond to nutrient change directly. This could have contributed to the overlap in community structure among sites, as these genera should have a greater influence on the overall community structure of the different sites. Benthic diatom genera that showed a positive correlation to nutrients were mostly associated with sites adjacent to the mariculture area, but they occurred only in very small numbers and were only present during a short period of time. It might be possible that these diatoms were not able to compete for nutrients under normal conditions but may proliferate only when excessive nutrients became available. For benthic diatoms, the general optimal stoichiometric ratio for C:N:P is 119:17:1. An N:P ratio < 13 indicates N-limitation, whereas an N:P ratio > 22 indicates P-limitation (Hillebrand and Sommer 1999). Among the sites, Tomasa, Lucero, and Victory were considered N-limited for benthic diatom growth, whereas Silaqui was P-limited. This may explain differences in the relative abundance of *Cylindrotheca*, the genus that had the largest contribution to dissimilarities among sites, and other species. The species- or genus-specific N:P requirements may explain site-specific differences in correlations between diatom genera and nutrient variability. More physiological behavior of these diatoms should be evaluated to verify this suggestion.

CONCLUSION

Results of the present study show the potential of marine benthic diatoms as indicators of ecosystem health based on changes in their diversity, richness, as well as correlation with nutrients and other environmental factors. In earlier studies, the response of freshwater benthic diatoms to nutrients was shown to be useful in assessing the status of freshwater ecosystems (Desrosiers *et al*. 2013). Whereas the present study provided insights into the response of community structure of benthic diatoms to the nutrient difference across sites, further studies in areas with strong persistent nutrient gradients will be important to enhance the understanding of the

effects of nutrient enrichment on the dynamics of benthic diatom assemblages and their spatio-temporal patterns. This knowledge is particularly wanting for nearshore tropical marine benthic ecosystems. Many coastal areas in Southeast Asia are currently facing tremendous developmental pressures, including mariculture expansion. Developing biological indicators for nutrient and other pollution effects is never this urgent.

Genus-level responses showed interesting patterns of community response to different nutrient conditions. However, species-level responses should be conducted to further explore a more specific basis for considering benthic diatoms as indicators of ecosystem status. This may be applied initially to species within genera that showed strong correlations with nutrients. However, interactive effects with other factors like irradiance and temperature should also be considered.

Finally, another finding of significance in this study is that diatom community structures on glass and polycarbonate were found to be similar in terms of their composition, diversity, and richness. Although differences in cell densities were notable, these were not consistent among sites. These results suggest that polycarbonate may be used as an alternative to glass in marine biofilm studies in the natural environment. Polycarbonate is more durable and is less likely to break compared to glass. It could also be cut into various shapes and sizes for convenient use and for simulating different physical substrates. Its potential use in pollution studies and assessment should be further explored.

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STATEMENT ON CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

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APPENDIX

Table I. Presence and absence of different genera in benthic diatom communities in different sites from Days 1–6.

Family	Genera (21)	Tomasa						Lucero						Silaqui						Victory					
		Glass			Polycarbonate			Glass			Polycarbonate			Glass			Polycarbonate			Glass			Polycarbonate		
		1	3	6	1	3	6	1	3	6	1	3	6	1	3	6	1	3	6	1	3	6	1	3	6
Pennate (14 genera)																									
Naviculaceae	*Navicula*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Haslea	x			x			x			x		x												
Bacillariaceae	*Nitzschia*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Cylindrotheca	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Bacillaria	x												x											
	Pseudonitzschia							x																	
Fragilariaceae	*Asterionellopsis*							x							x										
Thalassionemataceae	*Thalassionema*	x			x			x																	
	Thalassiothrix	x											x	x	x						x				
	Lioloma							x												x			x	x	x
Catenulaceae	*Amphora*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Pleurosigmataceae	*Pleurosigma*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Licmophoraceae	*Licmophora*		x			x	x	x			x	x		x	x	x									
Diploneidaceae	*Diploneis*		x			x		x				x													
Centric (7 genera)																									
Chaetocerotaceae	*Chaetoceros*															x			x	x					
	Bacteriastrum													x	x				x						
Skeletonemaceae	*Skeletonema*	x											x			x									
Coscinodiscaceae	*Coscinodiscus*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Thalassiosiraceae	*Thalassiosira*	x			x			x			x		x								x				
Rhizosoleniaceae	*Rhizosolenia*	x			x																				
Hemiaulaceae	*Eucampia*								x																

Table II. Comparison of benthic diatom assemblages in terms of mean (± SD) diatom cell densities, richness, and diversity indices between glass and polycarbonate in different sites at different time points using t-test (n = 3).

Indices	Site	Day 1		Day 3		Day 6	
		Glass	Polycarbonate	Glass	Polycarbonate	Glass	Polycarbonate
Cell density (cells cm^{-2})	Tomasa	1,619 ± 280*	515 ± 73.7	23,744 ± 747	31,195 ± 5,576	268,267 ± 45,658	322,133 ± 57,474
	Lucero	3,617 ± 844*	1,671 ± 52.4	225,227 ± 7471*	56,560 ± 6,961	464,533 ± 49,064*	239,733 ± 12,949
	Silaqui	484 ± 108	462 ± 149	21,525 ± 2,173*	5,226 ± 485	5,909 ± 664*	2,901 ± 290
	Victory	899 ± 193	926 ± 143	30,904 ± 25,781	113,813 ± 10,031*	24,272 ± 3,783	105,867 ± 7,163*
Richness	Tomasa	2.71 ± 0.07	3.21 ± 0.08*	1.98 ± 0.01	1.93 ± 0.04	1.60 ± 0.02	1.58 ± 0.02
	Lucero	2.45 ± 2.69	2.69 ± 0.01*	1.62 ± 0.01	1.83 ± 0.02*	1.54 ± 0.01	1.61 ± 0.01*
	Silaqui	3.25 ± 0.11	3.28 ± 0.17	2.00 ± 0.02	2.34 ± .03*	2.30 ± 0.03	2.51 ± 0.03*
	Victory	2.95 ± 0.09	2.93 ± 0.07	2.19 ± 0.56	1.72 ± 0.01	1.98 ± 0.03*	1.73 ± 0.01
Diversity	Tomasa	1.60 ± 0.07	1.56 ± 0.10	1.17 ± 0.02	1.11 ± 0.04	1.30 ± 0.01	1.41 ± 0.06*
	Lucero	1.19 ± 0.05	1.18 ± 0.02	0.93 ± 0.01	0.95 ± 0.02	1.24 ± 0.06*	1.01 ± 0.02
	Silaqui	1.33 ± 0.06	1.27 ± 0.02	1.31 ± 0.01	1.45 ± 0.06	1.47 ± 0.05	1.58 ± 0.01*
	Victory	1.29 ± 0.05	1.29 ±.0.10	1.19 ± 0.06	1.28 ± 0.03	1.30 ± 0.04*	1.07 ± 0.02

*Asterisks denote significantly higher mean cell density, richness or diversity index (t-test, $p < 0.05$) on glass or polycarbonate.

Table III. Comparison of mean (± SD) cell densities (cells cm^{-2}) of benthic diatom assemblages in different sites at different time points in each type of substrate using one-way ANOVA.

Substrate	Site*	Cell density (cells cm^{-2})**		
		Day 1	Day 3	Day 6
Glass	Tomasa	$1,619 \pm 280^{b2}$	$23,744 \pm 747^{b2}$	$268,267 \pm 45,658^{b1}$
	Lucero	$3,617 \pm 844^{a3}$	$225,227 \pm 7,471^{a2}$	$464,533 \pm 49,064^{a1}$
	Silaqui	484 ± 108^{c3}	$21,525 \pm 2,173^{b1}$	$5,909 \pm 664^{c2}$
	Victory	899 ± 193^{bc1}	$30,904 \pm 5,781^{b1}$	$24,272 \pm 3,783^{c1}$
Polycarbonate	Tomasa	515 ± 73.7^{b2}	$31,195 \pm 5,576^{bc2}$	$322,133 \pm 57,474^{a1}$
	Lucero	$1,671 \pm 52.4^{a3}$	$56,560 \pm 6,961^{b2}$	$239,733 \pm 12,949^{b1}$
	Silaqui	462 ± 149^{b3}	$5,226 \pm 485^{c1}$	$2,901 \pm 290^{d2}$
	Victory	926 ± 143^{ab2}	$113,813 \pm 10,031^{a1}$	$105,867 \pm 7,163^{c1}$

*Similar letters in superscript denote significant groupings among sites within each sampling day based on Tukey's test ($p < 0.05$).
**Similar numbers in superscript denote significant groupings among sampling time points within each sampling site based on Tukey's test ($p < 0.05$).

Table IV. Results of similarity percentage (SIMPER) analysis showing percentage contribution of different benthic diatom genera to dissimilarity among sites.

Taxon	Average dissimilarity	Contribution %	Cumulative %
Cylindrotheca	4.309	12.94	12.94
Amphora	3.727	11.19	24.13
Pleurosigma	3.525	10.58	34.71
Nitzschia	3.173	9.528	44.24
Navicula	3.134	9.409	53.65
Coscinodiscus	2.537	7.617	61.27
Licmophora	2.129	6.391	67.66
Lioloma	2.052	6.16	73.82
Chaetoceros	1.691	5.076	78.89
Thalassiothrix	1.375	4.129	83.02
Diploneis	0.9793	2.94	85.96
Skeletonema	0.931	2.795	88.76
Thalassiosira	0.9107	2.734	91.49
Haslea	0.7568	2.272	93.76
Eucampia	0.61	1.832	95.6
Asterionellopsis	0.3999	1.201	96.8
Thalassionema	0.3843	1.154	97.95
Bacillaria	0.3152	0.9463	98.9
Bacteriastrum	0.2269	0.6812	99.58
Rhizosolenia	0.07068	0.2122	99.79
Pseudonitzschia	0.06982	0.2096	100

Table V. Results of similarity percentage (SIMPER) analysis showing percentage contribution of different benthic diatom genera to dissimilarity among sampling days.

Taxon	**Average dissimilarity**	**Contribution %**	**Cumulative %**
Cylindrotheca	4.777	13.4	13.4
Amphora	3.934	11.04	24.44
Nitzschia	3.862	10.83	35.27
Pleurosigma	3.754	10.53	45.8
Navicula	3.571	10.02	55.82
Coscinodiscus	2.823	7.918	63.73
Licmophora	2.383	6.686	70.42
Lioloma	1.828	5.128	75.55
Chaetoceros	1.674	4.696	80.24
Thalassiothrix	1.395	3.913	84.16
Diploneis	1.018	2.857	87.01
Skeletonema	0.9526	2.672	89.69
Thalassiosira	0.9343	2.621	92.31
Haslea	0.7203	2.021	94.33
Eucampia	0.6079	1.705	96.03
Asterionellopsis	0.3855	1.081	97.11
Thalassionema	0.3795	1.065	98.18
Bacillaria	0.2847	0.7986	98.98
Bacteriastrum	0.2386	0.6693	99.65
Rhizosolenia	0.06384	0.1791	99.83
Pseudonitzschia	0.06212	0.1742	100

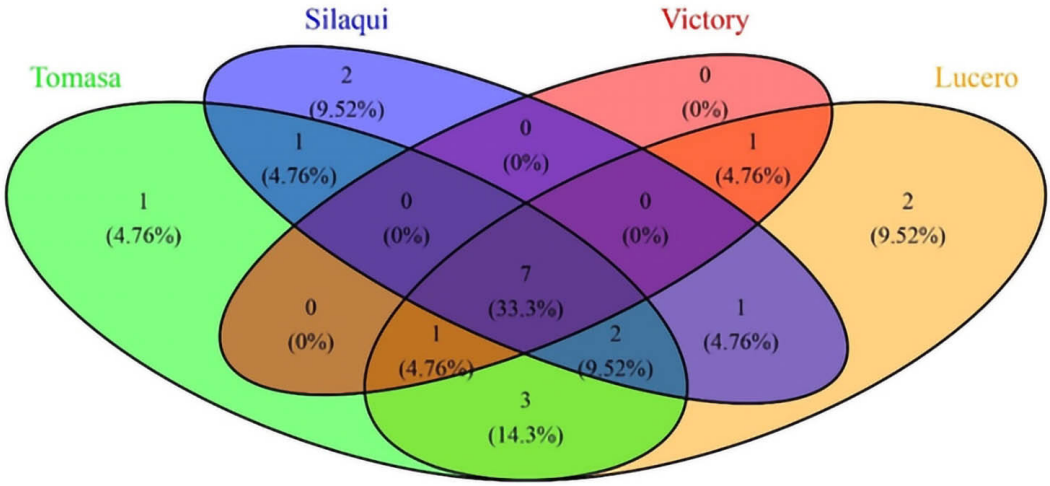

Figure I. Venn diagram showing the number of unique and shared diatom genera among different sites. Seven genera or ~ 33.3% of the benthic diatom genera are shared by all sites. The number of unique genera per site is indicated in sets that do not intersect.

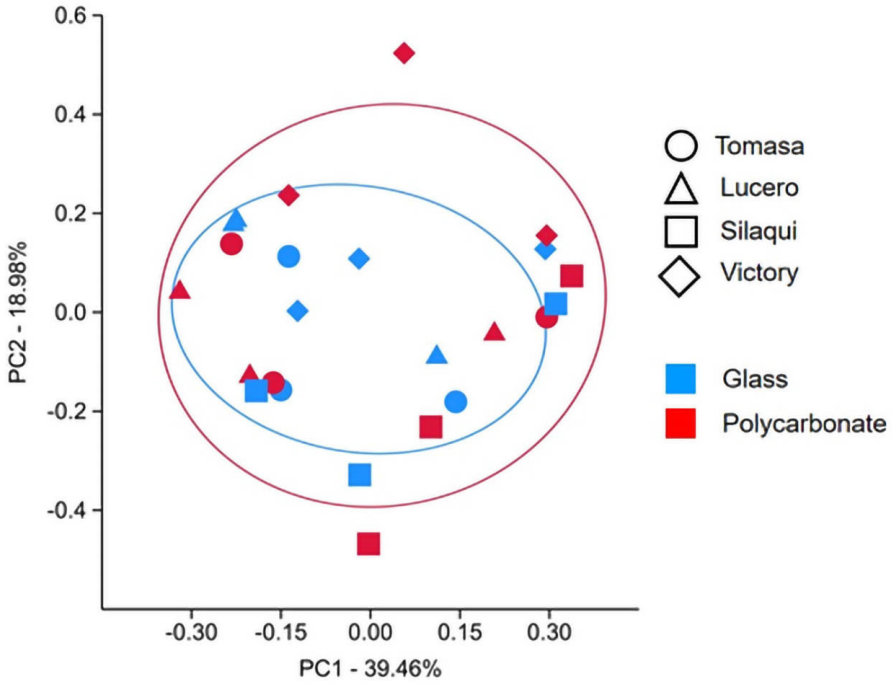

Figure II. Principal coordinate analysis (PCoA; ANOSIM R = –0.047, $p = 0.786$) showing significant overlaps of the community structure of benthic diatoms between glass slides and polycarbonate sheets in different sites. Each point represents average Hellinger-transformed data of mean total cell counts (density) of all diatom genera on each substrate type within a site on a given day of sampling.

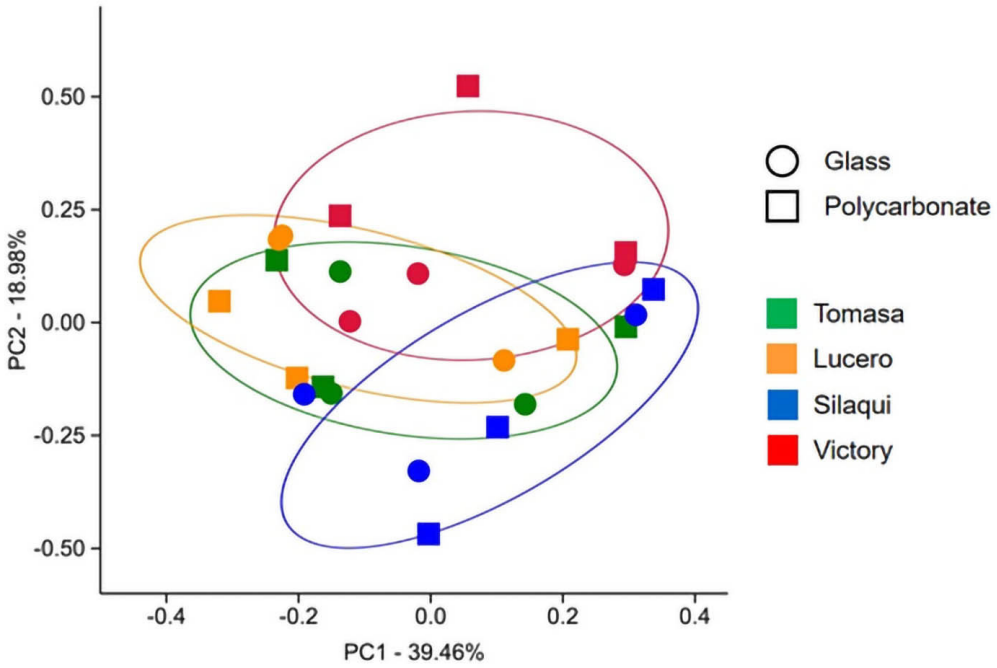

Figure III. Principal coordinate analysis (PCoA; ANOSIM R = 0.139, p = 0.052) showing partial overlaps of community structures of benthic diatom in different sites with data collected at different time points. Each point represents average Hellinger-transformed data of mean total cell counts (density) on each substrate type within a site on a given day of sampling.