


# Composition and abundance of benthic microalgae from the Estuarine Complex of Paranaguá Bay (southern Brazil) with special emphasis on toxic species

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## ABSTRACT

This study addressed the occurrence and distribution of benthic microalgae, with emphasis on toxic species, on different substrates (macroalgae, seagrass and sediments) along the outer, euhaline zone of the Estuarine Complex of Paranaguá Bay, southern Brazil, from July 2015 to May 2016. Canonical analysis of principal coordinates (CAP) was used to identify the potential environmental drivers of microphytobenthic assemblage structure. A total of 18 potentially toxic benthic microalgae species were reported, including 7 dinoflagellates, 9 cyanobacteria, one diatom and one euglenophyte. The abundance of all potentially toxic microalgae varied consistently along the investigated period. Dinoflagellates dominated the microalgae assemblage in July and September 2015. Mean densities on macrophytes ranged from 33.6 cells g<sup>-1</sup> in May 2016 to 775.6 cells g<sup>-1</sup> in September 2015, being positively correlated with dissolved nitrogen-nutrients, water transparency, salinity, and chlorophyll-*a*. *Prorocentrum cf. lima* was the most frequent and abundant dinoflagellate. Overall, the highest abundance of toxic species occurred in February 2016, when cyanobacterial mats dominated by *Lyngbya cf. aestuarii* were observed throughout the estuary associated with periods of heavier rainfall and higher turbidity (shallower Secchi depth). At that time, a selected microphytobenthic extract exhibited acute toxicity on nauplii of the brine shrimp *Artemia salina* in the laboratory.

**Descriptors:** Microphytobenthos community structure, Harmful microalgae, Acute toxicity, Cyanobacterial mats, Toxic dinoflagellates, Subtropical estuary.

## INTRODUCTION

Microphytobenthos (MPB) is represented by phototrophic bacteria (i.e., cyanobacteria) and eukaryotic algae, mainly diatoms and dinoflagellates, inhabiting sediment surface, and constitutes

an important component of estuarine ecosystems (Semcheski et al., 2016). These organisms play an active role in the uptake of inorganic and organic nutrients and contribute to the primary productivity of shallow areas as basal components of the food web (Underwood and Kromkamp, 1999; Riekenberg et al., 2017). They produce extracellular polymeric substances (EPS) forming biofilms, which increase sediment stability (Tolhurst et al., 2002). In addition to their functional and trophic importances, both community structure and composition of MPB might exhibit a high degree of patchiness

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(Orvain et al., 2012), contributing to overall diversity and richness of benthic communities. Variations in sediment grain size, tidal height, wave energy and the abundance of grazers can affect microphytobenthic distribution creating areas that can act as either “hotspots” or “coldspots” of productivity, nutrient recycling, and carbon sinking (Brustolin et al., 2014; Riekenberg et al., 2018). Understanding the patterns of spatial distribution and temporal dynamics of MPB is thus pivotal.

Several microalgal species belonging to different taxonomic groups, however, can produce potent toxins and bioactive chemical compounds, negatively affecting marine organisms and human health during events known as harmful algal blooms (HABs). Among them, cyanobacteria and dinoflagellates are the main groups of organisms involved in benthic HABs in shallow aquatic environments (Moestrup et al., 2009). While dinoflagellates comprise the majority of HAB-forming species in the marine environment, most toxic events reported in fresh or brackish-water systems have been associated with cyanobacterial blooms (Hallegraeff et al., 2003; Paerl and Huisman, 2009; Quiblier et al., 2013). In shallow, transitional zones like estuarine deltas and tidal flats, variations in sediment characteristics (e.g., grain size, interstitial nutrient concentrations), as well as environmental gradients in the water column (e.g., salinity, temperature, particulate and dissolved organic matter concentrations), ultimately determine the composition and cell abundance of benthic microalgae, including HABs species (Moncer et al., 2017). The dynamics of benthic-pelagic coupling can thus regulate both bloom initiation and development, and their assessment should be included in monitoring and management programs aiming at mitigating the impacts of benthic HABs in these ecologically and economically important coastal areas.

Among marine dinoflagellates, species belonging to the genus *Ostreopsis* are known to produce the most potent marine toxins known to date - the palytoxin and its analogues, ovatoxins (Hoppenrath et al., 2014). Species of *Gambierdiscus* and *Fukuyoa*, two taxonomically related genera, can also produce potent neurotoxins namely ciguatoxins and maitotoxins. Annually, these toxins are responsible for hundreds of cases of Ciguatera Fish Poisoning

(Lassus et al., 2016), the most frequent seafood-borne disease of nonbacterial origin worldwide. Other benthic dinoflagellates such as *Amphidinium*, *Coolia*, *Prorocentrum* and *Vulcanodinium* may be less commonly associated with impacts to human and ecosystem health. For instance, some benthic *Prorocentrum* species produce okadaic acid (OA) and dinophysistoxins (DTX), the causative agents of Diarrhetic Shellfish Poisoning (DSP) in humans (Hoppenrath et al., 2013; Nascimento et al., 2016), while some *Coolia* species have been shown to produce cooliatoxin, a yessotoxin analog with neurotoxic action (Holmes et al., 1995; Wakeman et al., 2015). Finally, dense blooms of epibenthic *Vulcanodinium rugosum* and *Amphidinium* spp. may also affect the pelagic compartment of coastal areas. Whereas *V. rugosum* produces the cyclic imines pinnatoxins, which are fast-acting neurotoxins (Hoppenrath et al., 2014), *Amphidinium* spp. produce a number of bioactive compounds (amphidinols and amphidinolides) with cytotoxic and/or hemolytic activity, and may cause fish mortalities during blooms (e.g. Murray et al., 2015; Moreira-González et al., 2019). Some species of cyanobacteria, in addition, can produce a variety of toxic secondary metabolites (cyanotoxins) with biological effects ranging from hepatotoxicity (microcystins, nodularins) to dermatotoxicity (lyngbyatoxins, aplysiatoxins), cytotoxicity (cylindrospermopsins) and neurotoxicity (anatoxins, saxitoxins) (Pearson et al., 2010; Lopes and Vasconcelos, 2011).

Recent increase in the abundance of benthic toxic microalgae - and its associated deleterious effects to benthic communities - has been linked to climate changes, caused by global warming and anthropogenic activities including the eutrophication of coastal systems and marine habitat destruction (Skinner et al., 2013; Moncer et al., 2017). Blooms of the benthic dinoflagellate *Ostreopsis* have become more frequent and intense in temperate waters (Berdalet et al., 2017). Moreover, an increase in the abundance of toxic benthic dinoflagellates from the genera *Prorocentrum* and *Ostreopsis* has been consistently observed in coral reef communities experiencing a decrease in coral abundance due to eutrophication and acidification, for instance (Skinner et al., 2013). Coastal environments are thus facing an additional threat as benthic HABs

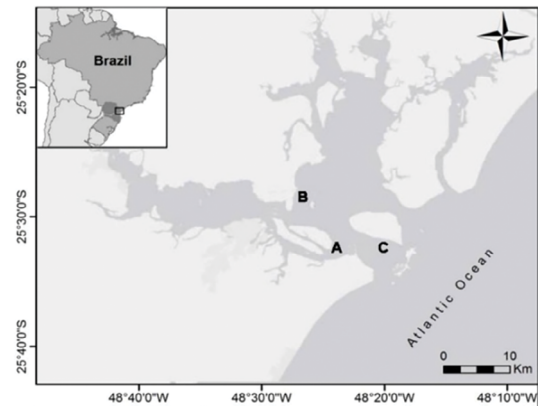
become more frequent, severe and spread over a broader geographic distribution, including temperate and subtropical areas.

The subtropical Estuarine Complex of Paranaguá Bay (ECPB) is one of the largest estuarine systems in Brazil. This estuary provides food and nursery to a great variety of marine life including economically important species of shrimp, crabs and fish, as well as species facing anthropogenic threats such as the “near threatened” Guiana dolphin (*Sotalia guianensis*) and the “endangered” green sea turtle (*Chelonia mydas*) (Lana et al., 2001). To date, ecological studies on harmful microalgae have been majorly focused on planktic species in the ECPB (e.g. Mafrá Júnior et al., 2006, 2014), so that the environmental and health risks associated with toxic benthic microalgae and cyanobacteria are largely neglected. Therefore, the main purpose of this study is to determine the temporal variation, from July 2015 to May 2016, in the composition and abundance of benthic microalgae - with emphasis on toxic species - growing on different substrates in the outer portion of the ECPB. The potential threats of toxic benthic microalgae to marine organisms and human health are evaluated.

## MATERIALS AND METHODS

### STUDY AREA

The ECPB is a tide-dominated estuarine system of the subtropical, southern Brazilian coast (25°30'S, 48°30'W; Figure 1), influenced by a mesothermic humid climate (~85% mean air humidity) (Lana et al., 2001). It is formed by four interconnected water bodies. While the Paranaguá and Antonina bays occupy 260km<sup>2</sup> along the east-west axis, the Laranjeiras and Pinheiros bays occupy 200km<sup>2</sup> along the south-north axis. The area is composed of a rich mosaic of nearly pristine habitats including mangroves swamps, salt marshes and extensive sand-muddy tidal flats. The environment can be divided into distinct sectors according to the salinity range: oligo- to mesohaline (inner), polyhaline (intermediate), and euhaline (outer) sectors (Lana et al., 2001). The mouth of the estuary is >10m deep and the bottom sediments are characterized by well-sorted fine to very fine sediments (grain size <0.062mm). The intermediate zone, also influenced by marine waters, represents the widest cross-section of the



**Figure 1.** Map of the study area and sampling sites along the Estuarine Complex of Paranaguá Bay, southern Brazil.

estuary, with depths ranging from 4 to 10m and the bottom mainly characterized by muddy sediments (Marone et al., 2005; Mizerkowski et al., 2012).

Weather conditions can vary according to two main climate seasons: dry (April-September), with mean salinity of 20-34 and water temperature of 18-25 °C, and rainy season (October-March), with mean salinity of 12-29 and water temperature of 23-30 °C. Mean annual precipitation is 3000mm (Mizerkowski et al., 2012). Freshwater input varies from 30% up to 170% of the annual mean during, respectively, the dry season and rainy seasons. Yet the annual precipitation does not exhibit a very pronounced dry-winter period like other humid subtropical areas around the world. The local hydrodynamics is controlled by a semidiurnal tidal range up to 2.2m. The system is partially mixed, with a residence time of 3.5 days and a mean freshwater runoff of up to 200 m<sup>3</sup> s<sup>-1</sup> (Lana et al., 2001; Marone et al., 2005).

### SAMPLING DESIGN AND SAMPLE COLLECTION

Sampling campaigns were performed approximately every three months, on July 1-2 (early austral winter) and September 23-24 of 2015 (early spring), and on February 23-24 (summer) and May 19-20 of 2016 (autumn). Three sampling sites were located at the outer (euhaline) sector of the ECPB: site A (25°32'S, 48°24'W), B (25°28'S, 48°27'W) and C (25°31'S, 48°19'W), with the latter closer to the mouth of the estuary (Figure 1).

In each sampling site, benthic microalgae were collected on three different substrates: bare sand

and two dominant macrophytes. This way, the entire diversity of microphytobenthic assemblage could be assessed, including epipelagic and epipsammic algal species inhabiting intertidal sediments, as well as epiphytic species associated with macrophytes. It was not possible to sample the same species of macrophyte at all occasions as their availability varied geographically and temporally throughout the studied period. The dominant macrophytes were mainly opportunistic chlorophycean algae such as *Ulva* and *Chaetomorpha* spp., while less frequent macrophytes included red seaweed members of the genera *Acanthophora* and *Hypnea*, as well as the seagrass *Halodule wrightii*. More specifically, macrophyte samples were composed of *Ulva lactuca*, *U. flexuosa*, *Ulva* sp. and *Hypnea spinella* in July 2015; *U. lactuca*, *Ulva* sp., *Acanthophora spicifera*, *Hypnea musciformis* and the seagrass *Halodule wrightii* in September 2015; *U. lactuca*, *Ulva* sp., *Chaetomorpha* sp., and *Lyngbya cf. aestuarii* in February 2016; and *U. lactuca*, *Ulva* sp., *Chaetomorpha* sp., *A. spicifera* and *H. wrightii* in May 2016. In all campaigns, six replicated samples were collected per substrate in each sampling site, representing 18 samples per site per month (216 samples in total).

Macrophytes samples of 5-16g wet weight were collected manually during low spring tide (about 0.1 to 0.3m deep), and placed into plastic bags containing 200mL of 0.22 $\mu$ m filtered seawater (FSW). In addition, a 2.5cm (diameter) corer was used to sample 1cm layers of superficial sediment, which were transferred to plastic bags as previously described. One extra sub-sample of sediment was collected from each site for determination of chlorophyll-*a* and pheophytin concentrations, and for granulometric analysis.

At each site, water samples were simultaneously collected from adjacent waters at 0.5m from the surface (sub-surface) and 0.5m from the bottom (mean depth ~ 3m) with a Van Dorn water sampler for nutrient analysis. Temperature and salinity were measured *in situ* with a calibrated COMPAC CTD profiler (JFE Advantech Co., Ltd.). Water transparency was estimated from a Secchi disk. Daily-averaged relative humidity, solar radiation, wind speed, and accumulated rainfall from the 15 days preceding each sampling campaign were obtained

from a semi-automatic meteorological station located near the ECPB, whose data were made available by the Brazilian National Meteorology Institute (INMET).

#### LABORATORY ANALYSES

Plastic bags containing water and macrophytes (or sediment) were vigorously shaken to detach microalgal cells from the substrate. The suspension was then passed through a 300 $\mu$ m Nitex mesh to eliminate larger particles, collected into amber glass flasks and preserved with Lugol's solution at 1% (final concentration).

Sample aliquots were observed prior to preservation for taxonomic identification. Both living and Lugol-preserved cells were photographed on an Olympus® BX-41 microscope using a Canon ELH 135 digital camera, under 400 $\times$  and 1000 $\times$  magnifications. Microalgal species were identified following Hallegraeff et al. (2003), Crispino and Sant'Anna (2006), Hoppenrath et al. (2014) and Lassus et al. (2016). Prior to cell enumeration all samples were manually homogenized for 1 min in order to remove excess sediment and, thereafter, 70mL aliquots of each preserved sample were dispensed into a 24 $\times$ 2cm (height  $\times$  diameter) glass tube. After allowing settlement of the larger sediment particles for 3 min, 1mL of each sample was drawn from the tube with a Pasteur pipette at 5cm depth (Brustolin et al., 2016), and microalgal cell numbers were counted under 200 $\times$  magnification using a Sedgewick-Rafter counting slide. The most abundant taxa, including non-toxic diatoms and selected cyanobacterium species collected in February 2016, were only counted in transects equivalent to 150 $\mu$ l of sample, which usually contained >300 cells of each taxon. In the case of free trichome-forming cyanobacteria, the average number of cells containing in 25 $\mu$ m long portions of the filaments was recorded (n=80 portions), and the total number of cells estimated by taking the number and length of trichomes into account. Fresh weight of each macrophyte was measured using a balance (Shimadzu®, model AY220) and the abundance of cells was then expressed as cells g<sup>-1</sup> (wet weight) of macrophyte, or as cells cm<sup>-2</sup> in the case of sediment samples, and posteriorly converted to relative (i.e. percent) abundances to allow comparison across different types of substrates.

Prior to nutrient analysis, seawater samples were filtered through Whatman-GF/C filters (45mm diameter, 0.8µm nominal pore size), which were stored at -18 °C until further treatment. The concentration of dissolved inorganic nutrients (Phosphate- $\text{PO}_4^{3-}$ , Ammonium- $\text{NH}_4^+$ , Nitrate- $\text{NO}_3^-$  and Nitrite- $\text{NO}_2^-$ ) was determined by spectrophotometric methods. Nitrate and nitrite concentrations were analyzed following the diazotization reaction; phosphate after the phosphorus blue reactions; and ammonium levels following the indophenol blue method (Grasshoff et al., 1983). Sediment samples for grain size determination were dried and treated with 10% hydrogen peroxide to digest the organic matter. Then, samples were dried again and weighed to obtain the total dry weight. Each grain size fraction ( $\geq 500$ ; 250-499; 125-249; 63-124; and  $\leq 63\mu\text{m}$ , or mud) was sieved, individually weighed, and expressed as a percentage of the total dry weight. The statistical parameters obtained from the granulometric analyses were calculated with the SysGran 3.0 software (Camargo, 2006).

#### ARTEMIA SALINA SURVIVAL TEST WITH A MICROPHYTOBENTHIC EXTRACT

Cyanobacterial mats, composed predominantly of *Lyngbya cf. aestuarii*, were manually collected at site A in February 2016. Prior to the preparation of the extract, a 5mL aliquot of the cyanobacterial mat sample was taken and stored in iodine Lugol solution (1%) for further counting of MPB cells. Then, approximately 5-10g of *L. cf. aestuarii*-dominated mats were disrupted using a sonic dismembrator (130W; Cole Parmer CPX130, USA) for 2 min at 50% duty cycle, to release the cellular content. An aliquot of the extract was observed under the microscope to assure complete cell disruption. The sonicated extract was then diluted 8 times and filtered onto Millipore 0.22µm pore syringe filters to remove cell debris. Due to dilution before filtration, the maximum concentration tested was equivalent to 12.5% of the original homogenate. Successive dilutions were prepared in FSW and six concentrations corresponding to decreasing cell densities of *Lyngbya* were thus tested: 12.5% (1980 cell-eq.  $\text{mL}^{-1}$ ), 6.25% (990 cell-eq.  $\text{mL}^{-1}$ ), 3.12% (495 cell-eq.  $\text{mL}^{-1}$ ), 1.56% (248 cell-eq.  $\text{mL}^{-1}$ ), 0.78% (124 cell-eq.  $\text{mL}^{-1}$ ) and 0.33% (62 cell-eq.  $\text{mL}^{-1}$ ), in addition to a control treatment (FSW only). For each treatment

tested, 4.5mL of the solution were placed into wells of 6-well culture plates, in triplicate. Ten ( $\pm 2.8$ , standard deviation) 5-day-old *Artemia salina* larvae, previously eclosed from cysts under light and gentle aeration, were placed into each well. Survival was checked after 48h of exposure at 25 °C in darkness. Larvae were considered dead only when no internal or external movement was observed under a stereoscope during 30s. The toxicity results were expressed as the equivalent abundance of cyanobacteria (cell-eq.  $\text{mL}^{-1}$ ) causing 50% of mortality ( $LC_{50}$ ). The non-observed-effect concentration (NOEC) and the lowest-observed-effect concentration (LOEC) were also determined.

#### DATA ANALYSIS

Before averaging the values for each sampling month, and prior to the statistical analysis, zero values were attributed whenever a given microalgal species exhibited cell abundance below the detection limit of the counting method (i.e., 13 cells  $\text{cm}^{-2}$  or 4-13 cells  $\text{g}^{-1}$ , depending on the weight of macrophytes sampled). In order to visualize the spatial structure of both overall and potentially toxic microphytobenthic assemblages over four different months, non-metric multi-dimensional scaling (n-MDS) (Anderson and Willis, 2003) analyses based on the Bray-Curtis dissimilarity matrices were carried out. To evaluate the significance of differences between groups of samples observed in n-MDSs, permutational multivariate analyses of variance (PERMANOVA, Anderson, 2001) based on the Bray-Curtis dissimilarity matrices were performed. Because of the restricted number of possible permutations in pairwise tests, *p*-values were obtained from Monte Carlo samplings based on 999 permutations (Anderson and Robinson, 2003).

Canonical analysis of principal coordinates (CAP) was further performed to identify possible correlations between the environmental variables and either toxic microalgae or the overall microphytobenthic assemblage. Environmental variables were standardized (z-scores) and microalgal relative abundances were transformed using the arcsine square root. ANOVAs were then performed to evaluate the significance of the canonical axes. All analyses were performed in R environment using the 'vegan' package (Oksanen et al., 2016).



## RESULTS

### ENVIRONMENTAL VARIABLES

The average grain size ranged from 156 $\mu\text{m}$  in May to 199 $\mu\text{m}$  in July (Table 1), whereas average organic matter contents varied from 1.12% in July to 3.13% in May. Carbonate content varied between 0.99% in July and 3.94% in May. There was no clear temporal pattern for the variation of chlorophyll-*a* concentrations in the sediment over the studied period, with mean values ranging from 7.60 $\mu\text{g mm}^{-3}$  in May to 13.0 $\mu\text{g mm}^{-3}$  in July (Table 1).

Seawater temperature ranged from 20.9 °C in July to 28.1 °C in February, whereas the highest averaged salinity value (32.0) was recorded in May and the lowest (23.7) in February. Secchi disk transparency varied from 1.20m in February and May to 1.70m in July (Table 1). The highest averaged ammonium (13.2 $\mu\text{mol L}^{-1}$ ) concentration was recorded in September and the lowest (10.5 $\mu\text{mol L}^{-1}$ ) in May. Average nitrate and nitrite concentrations varied from 0.94 $\mu\text{mol L}^{-1}$  in February to 2.36 $\mu\text{mol L}^{-1}$  in July, and from 0.18 $\mu\text{mol L}^{-1}$  in September to 1.02 $\mu\text{mol L}^{-1}$  in May, respectively. Phosphate levels concentrations, on average, ranged from 0.07 $\mu\text{mol L}^{-1}$  in February to 0.31 $\mu\text{mol L}^{-1}$  in September (Figure 2).

**Table 1.** Mean values of physical-chemical parameters measured in the seawater and sediment from the ECPB during the period of July 2015 to May 2016: water temperature (T); salinity (Sal); Secchi transparency (SD); chlorophyll-*a* concentration (Chl. *a*); mean grain size (Grain S.); organic matter content (OMC); and calcium carbonate content (CaCO<sub>3</sub>).

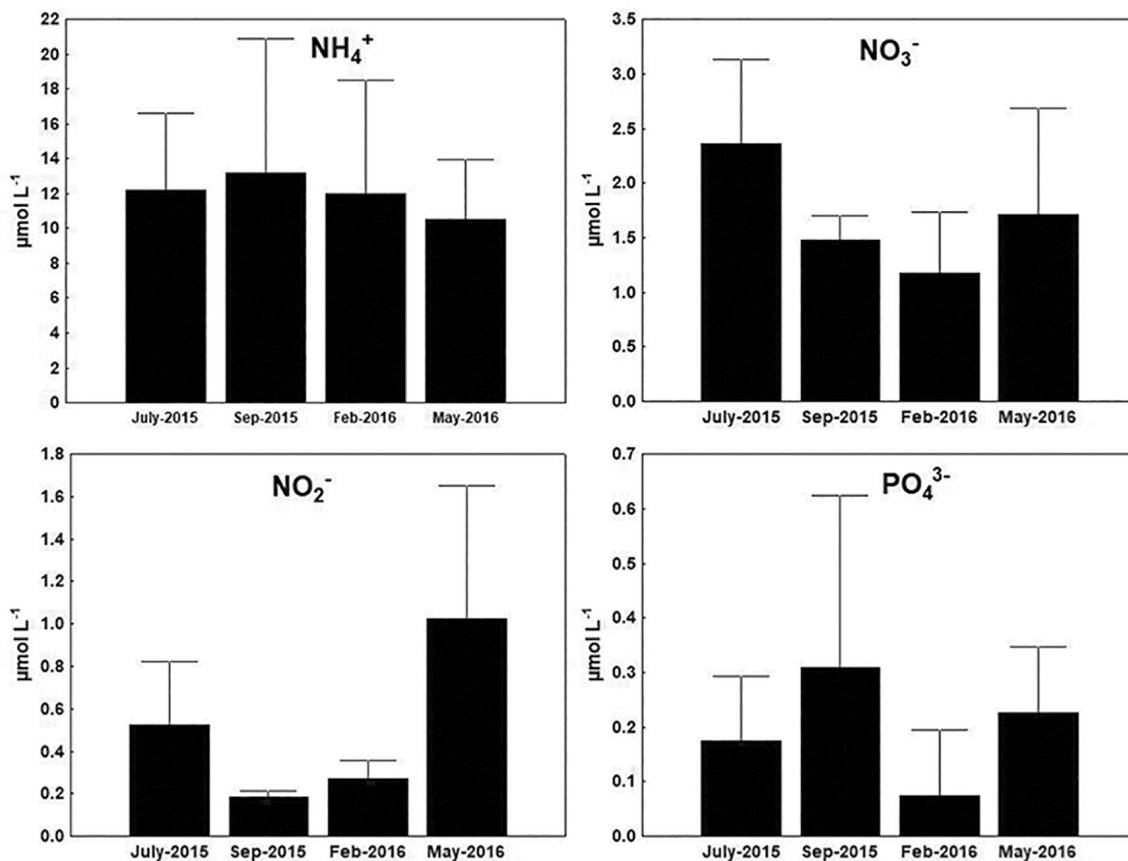
|   | July<br>2015 | Sept.<br>2015 | Feb.<br>2016 | May<br>2016 |
|---|--------------|---------------|--------------|-------------|
| <b>Water column</b>                     |              |               |              |             |
| T (°C)                                  | 20.9         | 24.8          | 28.1         | 21.6        |
| Sal                                     | 30.5         | 31.5          | 23.7         | 32.0        |
| SD (m)                                  | 1.70         | 1.30          | 1.20         | 1.20        |
| <b>Sediment</b>                         |              |               |              |             |
| Chl. <i>a</i> ( $\mu\text{g mm}^{-3}$ ) | 13.0         | 12.5          | 11.6         | 7.60        |
| Grain S. ( $\mu\text{m}$ )              | 199          | 172           | 173          | 156         |
| OMC (%)                                 | 1.12         | 1.31          | 1.59         | 3.13        |
| CaCO <sub>3</sub> (%)                   | 0.99         | 2.26          | 2.35         | 3.94        |

Among the relevant meteorological variables, solar radiation - measured as the average value over the 15 days preceding the sampling date - was always greater than 300MJ m<sup>-2</sup> d<sup>-1</sup>, with the minimum value (360MJ m<sup>-2</sup> d<sup>-1</sup>) recorded in July and the maximum (891MJ m<sup>-2</sup> d<sup>-1</sup>) in February. The highest averaged wind speeds were 2.64m s<sup>-1</sup> in February and 2.53m s<sup>-1</sup> in September, whereas the lowest value (2.08m s<sup>-1</sup>) was recorded in July (Table 2). Furthermore, relative air humidity and precipitation followed the typical characteristics of a humid subtropical ecosystem, with high relative humidity throughout the year and intense rainfall during the rainy-summer period (here represented by February sampling campaign). The highest 15d accumulated precipitation was 339mm, recorded in February, and the lowest was 20.0mm, registered in July (Table 2).

### TEMPORAL VARIATION OF THE MICROPHYTOBENTHIC ASSEMBLAGE WITH EMPHASIS ON TOXIC SPECIES

The MPB was dominated by non-toxic diatoms, both in terms of richness and cell density (>90% of the total abundance) (Figure 3 and Appendix A). The most frequent and abundant were the pennate species belonging to the *Pleurosigma/Gyrosigma* complex. Among the centric diatoms, the marine tychoplanktic *Paralia sulcata* was the most common species. Less abundant components of the microphytobenthic community included the euglenophyte *Euglenaformis proxima* and non-toxic benthic dinoflagellates such as *Amphidinium bipes*, *Amphidiniopsis* sp., *Ankistrodinium semilunatum*, *Sinophysis* sp. and *Prorocentrum* cf. *fukuyoi* (Appendix A). Within each of the major groups of benthic microalgae, cell abundance of cyanobacteria was markedly higher in February, while euglenophytes were more abundant in May, and dinoflagellates in July and September, relative to the other months. Diatoms were slightly less abundant in September (Figure 3 and Appendix A), but always dominant compared to other algal groups.

Eighteen potentially toxic species of microalgae were found within the MPB assemblage, including nine species of cyanobacteria, seven of dinoflagellates, one diatom (*Pseudo-nitzschia* sp.) and one



**Figure 2.** Dissolved nutrient concentrations measured along the Estuarine Complex of Paranaguá Bay, southern Brazil.

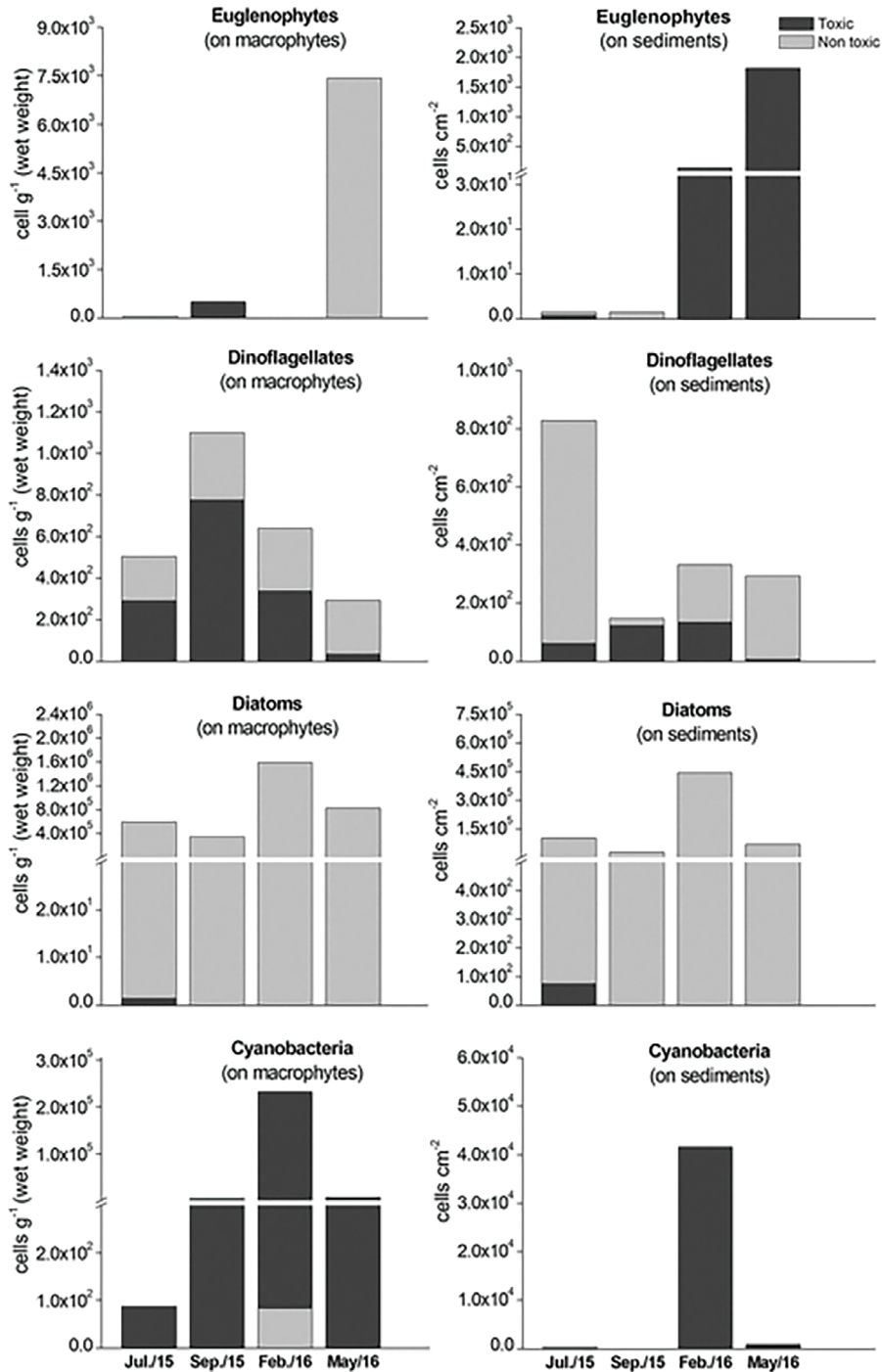
**Table 2.** Meteorological variables measured in the study area. Mean values calculated from the records of 15 days preceding the sampling date (accumulated values in the case of rainfall).

|  | July 2015 | Sept. 2015 | Feb. 2016 | May 2016 |
|--|-----------|------------|-----------|----------|
| Rainfall (mm)  | 20.0      | 54.2       | 339       | 49.2     |
| Humidity (%)   | 84.9      | 85.9       | 84.3      | 85.1     |
| Wind speed ( $\text{m s}^{-1}$ )                       | 2.08      | 2.53       | 2.64      | 2.18     |
| Solar radiation ( $\text{MJ m}^{-2} \text{day}^{-1}$ ) | 360       | 636        | 891       | 764      |

euglenophyte (*Euglenaria clavata*) (Appendix A). Potentially toxic cyanobacteria were represented by seven genera (*Anabaena*, *Calothrix*, *Chroococcus*, *Coelosphaerium*, *Lyngbya*, *Merismopedia*, *Oscillatoria* and *Phormidium*); whereas dinoflagellates were represented by four genera (*Amphidinium*, *Coolia*, *Dinophysis* and *Prorocentrum*) (Figures 4 and Appendix A). Fourteen of the potentially

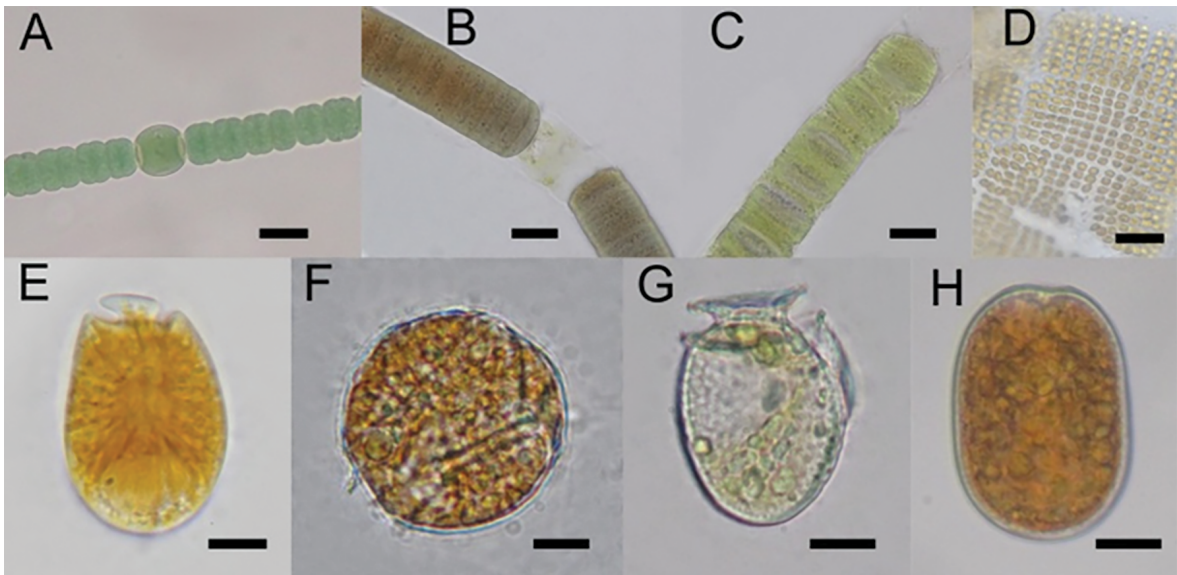
toxic species were typically benthic, while other four were planktic species eventually found on the sediment and macrophytes, including the dinoflagellates *Dinophysis cf. acuminata* and *Prorocentrum minimum*, the diatom *Pseudo-nitzschia sp.*, and the cyanobacteria *Coelosphaerium sp.* (Appendix A). These were included in our analysis assuming that they would be readily available to the benthic food web. Nevertheless, typical benthic species were always dominant, contributing with 79.5% (July in station C) to 99.8% (February in station A) of the total number of potentially toxic microalgal cells living in close association with the substrate.

The structure of the microphytobenthic assemblage - both for overall MPB and potentially toxic microalgae - varied significantly over time and between the two types of substrate during the investigated period ( $p=0.001$  in all cases). In addition, in both cases, there were significant interactions between the substrate type and time ( $p=0.001$ ),



**Figure 3.** Temporal variation of the cell abundance among the main groups of benthic microalgae collected on different substrates in the ECPB from July 2015 to May 2016. Note different y-axis scales.





**Figure 4.** Light-microscopy photomicrographs of some distinctive potentially toxic microalgal species found in the study area: (A) *Anabaena* sp.; (B-C) *Lyngbya* cf. *aestuarii*; (B) Vegetative trichome; (C) Reproduction of short trichome, showing the formation of hormogonia by cell division; (D) *Merismopedia elegans*; (E) *Amphidinium operculatum*; (F) *Coolia malayensis*; (G) *Dinophysis* cf. *acuminata*; (H) *Prorocentrum* cf. *lima*. Scale bars; (A-C): 5 $\mu$ m; (D-H): 10 $\mu$ m.

suggesting that the dissimilarity observed in assemblage structure over time is dependent on the substrate type (Figure 3 and Table 3). Among the potentially toxic species, cyanobacteria exhibited the highest cell densities, followed by euglenophytes and dinoflagellates (Figure 5).

Considering all macrophyte samples, cell abundance of potentially toxic cyanobacteria ranged from undetectable levels to 1,456,500 cells g<sup>-1</sup> (monthly average during the studied period: 60,300 cells g<sup>-1</sup>), and represented, on average, 99.4% of the total cell abundance of potentially toxic microalgae and 6.6% of the total benthic microalgal community. Maximum cell densities were registered in February (average: 231,600 cells g<sup>-1</sup>). The other groups were much less abundant. Cell density of potentially toxic dinoflagellates, for instance, ranged from undetected to 5200 cells g<sup>-1</sup> (monthly average: 360 cells g<sup>-1</sup>), with maximums in September (average: 780 cells g<sup>-1</sup>). They represented 0.6% of the total cell density of potentially toxic microalgae and only 0.04% of the total benthic microalgal community. For potentially toxic euglenophytes, cell abundance ranged from undetected to 21,900 cells g<sup>-1</sup> (monthly average: 1900 cells g<sup>-1</sup>), with higher values registered in May (average: 4900 cells g<sup>-1</sup>). Finally, the potentially toxic diatom *Pseudo-nitzschia* sp. was detected only

in July and at very low cell abundance (maximum 52 cells g<sup>-1</sup>; Appendix A).

A peak in the abundance of potentially toxic benthic microalgae was observed in February, when blooms of cyanobacteria occurred (Figure 3). The cyanobacteria *L. cf. aestuarii* was the dominant species at that time with an average cell density of 51,500 cells g<sup>-1</sup>, accounting for 80.5% of the toxic microalgal abundance and 9.8% of the overall abundance in MPB. Widespread, short mats of cyanobacteria, dominated by *L. cf. aestuarii*, were observed in this area of the estuary. Other relatively abundant cyanobacterial species, such as *Anabaena* sp., *Merismopedia elegans* and *Phormidium* spp., represented 10.8%, 1.7% and 1.6% of the toxic microalgal abundance in summer, respectively (Figure 5 and Appendix A). In turn, the potentially toxic dinoflagellates were dominant in July and September. The highest density of *Prorocentrum* cf. *lima*, the most frequent and abundant species among toxic dinoflagellates, was registered in July (1900 cells g<sup>-1</sup>) (Figure 5). Although the maximum abundance of dinoflagellates has occurred in July, accounting for 78.1% of contribution to the total abundance of potentially toxic benthic microalgae, their relative contribution to the total abundance of microphytobenthos was very limited (0.09%) at that time (Figure 3). Moreover, toxic

**Table 3.** Permutational multivariate analysis of variance (PERMANOVA) of the microphytobenthic assemblage, and potentially toxic microalgae among substrates and sampling months.

|           | Microphytobenthic assemblage |         |          |          | Toxic microalgae |         |          |          |
|-----------|------------------------------|---------|----------|----------|------------------|---------|----------|----------|
|           | df                           | SS      | Pseudo-F | p (perm) | df               | SS      | Pseudo-F | p (perm) |
| Time      | 3                            | 1.883e5 | 22.922   | 0.001    | 3                | 1.515e5 | 19.444   | 0.001    |
| Substrate | 1                            | 30303   | 11.065   | 0.001    | 1                | 35953   | 13.834   | 0.001    |
| Tm x Sb   | 3                            | 80867   | 9.842    | 0.001    | 3                | 29618   | 11.397   | 0.001    |
| Res       | 208                          | 5.696e5 | -        |          | 208              | 5.405e5 | -        |          |
| Total     | 215                          | 8.818e5 | -        |          | 215              | 8.413e5 | -        |          |
| Pair-wise | July 2015                    |         |          |          | July 2015        |         |          |          |
|           | Stations                     | t       | p (perm) | p (MC)   | Stations         | t       | p (perm) | p (MC)   |
|           | sed-mac                      | 2.264   | 0.004    | 0.004    | sed-mac          | 1.897   | 0.002    | 0.004    |
|           | Sept. 2015                   |         |          |          | Sept. 2015       |         |          |          |
|           | Stations                     | T       | p (perm) | p (MC)   | Stations         | t       | p (perm) | p (MC)   |
|           | sed-mac                      | 3.158   | 0.001    | 0.001    | sed-mac          | 3.179   | 0.001    | 0.001    |
|           | Feb. 2016                    |         |          |          | Feb. 2016        |         |          |          |
|           | Stations                     | t       | p (perm) | p (MC)   | Stations         | t       | p (perm) | p (MC)   |
|           | sed-mac                      | 4.572   | 0.001    | 0.001    | sed-mac          | 4.547   | 0.001    | 0.001    |
|           | May 2016                     |         |          |          | May 2016         |         |          |          |
|           | Stations                     | t       | p (perm) | p (MC)   | Stations         | t       | p (perm) | p (MC)   |
|           | sed-mac                      | 3.216   | 0.001    | 0.001    | sed-mac          | 4.712   | 0.001    | 0.001    |

dinoflagellates and cyanobacteria had a higher contribution to overall cell abundances in macrophyte relative to sediment samples (Figure 3).

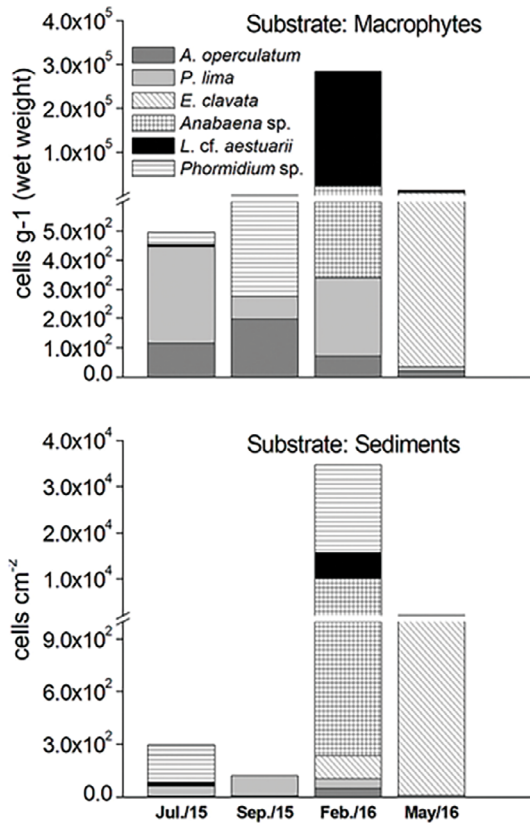
As revealed by CAP analysis, the abundance of *L. cf. aestuarii* and other dominant cyanobacteria was strongly associated with higher rainfall in summer. Likewise, cell density of dominant dinoflagellates, including toxic species (e.g., *A. operculatum* and *P. cf. lima*), exhibited a moderate, positive correlation with water transparency and salinity. In both cases, CAP explained a significant amount of variation in assemblage structure ( $R^2=50.9\%$  and  $R^2=43.2\%$  for overall MPB and potentially toxic microalgae, respectively) (Figures 6 and 7). In addition, a few species among the dominant potentially toxic MPB exhibited some degree of substrate preference. The cyanobacteria *L. cf. aestuarii* as well as the dinoflagellates *A. operculatum* and *P. cf. lima* were relatively more abundant on macrophytes than sediments, while *Phormidium* sp., in contrast, was generally more abundant on sediments, except in September (Figure 5).

#### ACUTE TOXICITY OF A MICROPHYTOBENTHIC EXTRACT TO ARTEMIA SALINA LARVAE

The extract of *Lyngbya cf. aestuarii*-dominated mats was acutely toxic to *Artemia salina* larvae, with a median lethal concentration ( $LC_{50}$ ) equivalent to 830 cells mL<sup>-1</sup> after 48h of exposure. At that time, the lowest-observed-effect concentration (LOEC) was equivalent to 62 cells mL<sup>-1</sup>, which corresponded to the lowest exposure level tested in this bioassay.

#### DISCUSSION

This study reported the occurrence of potentially toxic benthic microalgae in the Estuarine Complex of Paranaguá Bay (ECPB), southern Brazil, associated with different substrates such as green algae, red seaweed, seagrass and fine sediment. However, microphytobenthos (MPB) was largely dominated - in terms of richness and abundance - by non-toxic pennate diatoms, as previously reported in the ECPB (Fonseca et al., 2013) and in other estuaries worldwide (Semcheski et al., 2016).



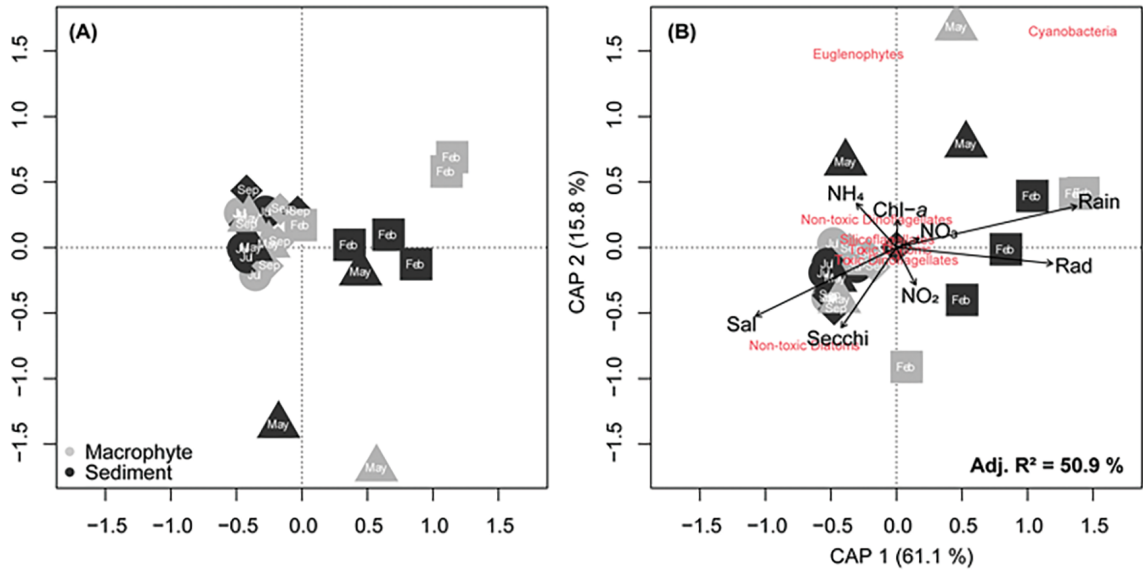
**Figure 5.** Spatial and temporal variation of the cell abundance among the dominant species of potentially toxic benthic microalgae collected on different substrates in the ECPB from July 2015 to May 2016.

In this large estuary, high abundance of non-toxic benthic microalgae, in especial diatoms, should therefore support high secondary productivity by benthic consumers like bivalves, crabs, fish, gastropods, isopods and polychaetes (Choy et al., 2008), as well as pelagic consumers (e.g. zooplankton) via resuspension of benthic microalgae into the water column (Modéran et al., 2012). In contrast, sediments and macrophytes from the ECPB sheltered a relatively low species richness and abundance of benthic dinoflagellates compared to that of marine environments from different parts of the globe, including other Brazilian regions (Nascimento et al., 2012, 2016; Chomérat et al., 2018). These findings agree with previous reports for other brackish water-dominated estuaries, where heavier freshwater inflows tend to favor diatoms, cyanobacteria and green algae to the detriment of dinoflagellates (Fonseca et al., 2013; Maggi et al., 2017).

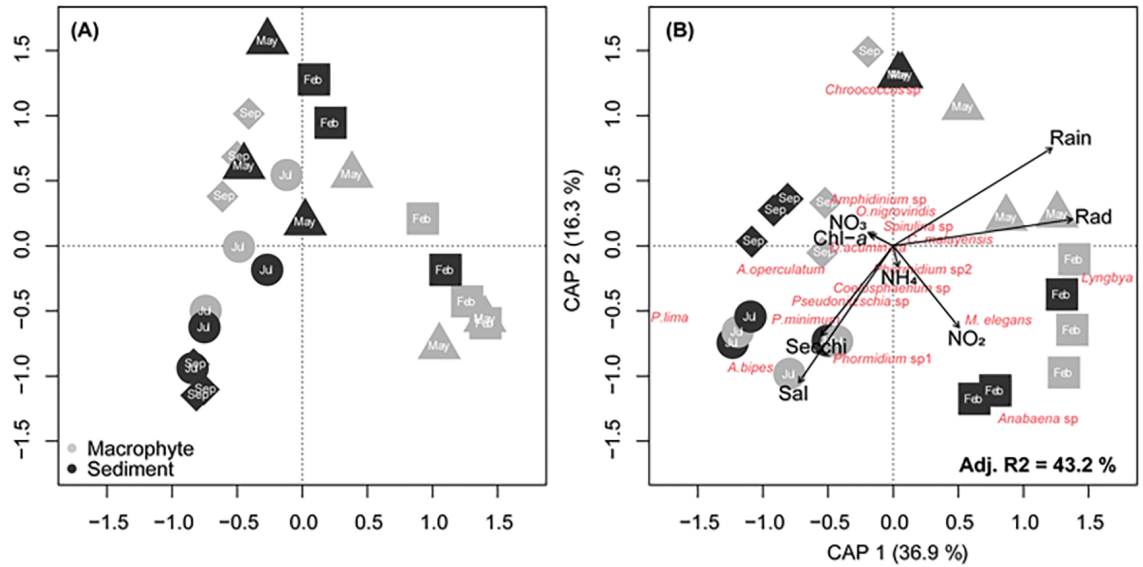
The results described in this study provide a first glance at the temporal variability in diversity and abundance of potentially toxic MPB taxa occurring in this large and ecologically relevant subtropical estuary. Local assemblages of potentially toxic MPB were dominated by low densities of dinoflagellates in winter (July-September), and by high densities of cyanobacteria in summer (February). The dominant cyanobacteria, mainly *L. cf. aestuarii*, exhibited high abundance in February and were correlated with heavier rainfall, lower salinity and higher turbidity (shallower Secchi depths) typical of summer in this subtropical area. In fact, cyanobacteria can tolerate wide and abrupt changes in salinity and temperature, and are able to survive under low light conditions (Paerl et al., 2000). This provides a competitive advantage in estuarine environments subjected to intense discharge of freshwater and the associated high loads of particles in suspension, as observed under regular summer conditions in the ECPB (Lana et al., 2001; Funari and Testai, 2008). Nevertheless, no other study has previously focused on benthic cyanobacteria in the ECPB area to support the temporal pattern indicated herein.

When favorable conditions prevail, such as high temperature, high pH, and increased availability of nutrients, especially those artificially released by anthropogenic activities (Chorus and Bartram, 1999), cyanobacteria can form superficial blooms in estuaries. Mostly in summer, the ECPB receives intense continental and urban discharges from extensive drainage basins, retaining sediments, nutrients, organic compounds, heavy metals and other contaminants (Choueri et al., 2009; Cabral et al., 2018). Although the concentrations of nutrients, as determined in the water column adjacent to the sampling sites, were not as high throughout the present study, moderate concentrations of dissolved inorganic nitrogen such as ammonium were recorded in February. Furthermore, concentrations of inorganic phosphorus and nitrogen in the ECPB, including those measured in shallow areas of intertidal flats and in the sediments, are often higher than those measured in the present study (e.g., Mafra Junior et al., 2006; Fonseca et al., 2013).

Contrary to cyanobacteria, the development of benthic dinoflagellates - and their preferred host macrophytes - requires periods of greater



**Figure 6.** Constrained and unconstrained multidimensional ordination of the Bray-Curtis dissimilarities in overall microphytobenthic assemblage across substrates and time (sampling months). (A) Unconstrained non-metric multidimensional ordination n-MDS; (B) Constrained analysis of principal coordinates CAP – arrows' length is proportional to the variation in community explained by the environmental variables; Macrophytes in light gray, Sediment in dark gray.



**Figure 7.** Constrained and unconstrained multidimensional ordination of the Bray-Curtis dissimilarities in potentially toxic microalgae across substrates and time (sampling months). (A) Unconstrained non-metric multidimensional ordination n-MDS; (B) Constrained analysis of principal coordinates CAP - arrows' length is proportional to the variation in community explained by the environmental variables; Macrophytes in light gray, Sediment in dark gray. Only the most abundant species are shown (i.e., those contributing to 70% of the total relative abundance).

hydrological stability (Vila et al., 2001). Overall, benthic dinoflagellates are more abundant under relatively stable water column conditions, which reduce losses due to turbulence and runoff (Vila et al., 2001;

Richlen and Lobel, 2011). Likewise, the highest cell abundances of various dinoflagellates, including the potentially toxic species *Prorocentrum cf. lima* and *Amphidinium operculatum*, were recorded on



opportunistic green macroalgae (*Ulva* spp.) blooming mainly in the most sheltered sampling sites of the ECPB in July. During this drier period, higher water transparency, lower runoff contribution and higher salinity can all stimulate the growth of dinoflagellates inside the estuary.

Moderate abundances of *P. cf. lima*, however, were also registered in February, during summer. At that time, cells were sampled from mats dominated by the benthic filamentous cyanobacterium *L. cf. aestuarii*, confirming the importance of macrophytic substrates for benthic dinoflagellates. Moreover, these findings also indicate that the low salinities (19-28) recorded during that time in the ECPB did not affect the development of *P. cf. lima* over the study area, in accordance to laboratory experiments showing that a local toxigenic strain of *P. cf. lima* was tolerant to abrupt salinity changes (Sobrinho and Mafra, 2016). Therefore, besides occurring throughout the year, it is plausible to assume that *P. cf. lima* inhabits an even larger estuarine area than that covered by the present study, increasing the risk for toxin transfer across the benthic food webs.

*Prorocentrum cf. lima* was the most frequent and abundant toxic dinoflagellate in this study. This species is widely distributed in both temperate and tropical waters, and can be an important source of the diarrhetic toxins okadaic acid (OA) and dinophysistoxins (DTXs) in coastal environments (Foden et al., 2005). A *P. cf. lima* strain isolated from the ECPB has been recently shown to produce moderate concentrations of both OA and DTX-1 (Moreira-González et al., 2018), indicating that the species could be thus an important source of toxins to the benthic food web in this region. The average cell densities of *P. cf. lima* found on macrophytes (up to 1860 cells g<sup>-1</sup> fresh weight) and on sediments (up to 320 cells cm<sup>-2</sup>) in the present study are within the range reported for this species in other coastal areas worldwide (Nascimento et al., 2005; Maranda et al., 2007; Takahashi et al., 2008). In fact, cells of *P. lima* complex were previously recorded (as *Prorocentrum* sp.) at even higher cell abundances (up to 3570 cells cm<sup>-2</sup>) in sediment samples from the ECPB (Brustolin et al., 2014). *Prorocentrum* sp. was another benthic dinoflagellate found on sediment (up to 301 cells cm<sup>-2</sup>) and macrophytes (up to 601 cells g<sup>-1</sup>) in the

present study. One specimen was isolated and cultured in parallel to this study. Toxicological examination of concentrated *Prorocentrum* sp. extracts indicated high acute toxicity to mice, even though no diarrhetic toxins have been detected in the same strain (Moreira-González et al., 2019).

Benthic dinoflagellates have proved to be important sources of marine toxins. Most potentially toxic species of benthic dinoflagellates found in the present study (e.g., *P. cf. lima*, *A. operculatum* and *Coolia malayensis*) are frequently reported over a broad range of different marine ecosystems worldwide, and may produce a wide variety of toxic compounds (Berdalet et al., 2017). Some strains of *Amphidinium* spp., including *A. operculatum*, produce compounds with hemolytic and antifungal properties (amphidinols and amphidinolides), which are potentially toxic to fish (Yasumoto et al., 1987; Espiritu et al., 2017). Similarly, *Coolia* spp. can also elicit cytotoxic and hemolytic effects, as shown by *in vitro* bioassays (Karafas et al., 2015; Leaw et al., 2016). Although there is no report of toxic effects on humans associated with *Coolia*, toxicological effects have been observed in mice (Paz et al., 2008; Korsnes et al., 2014; Karafas et al., 2015; Moreira-González et al., 2019). Potentially causative compounds may include the cooliatoxins, a yessotoxin analog first described in some strains of *Coolia tropicalis* Faust (Holmes et al., 1995). Recently, five yessotoxin analogs were chemically characterized from *C. malayensis* from Okinawa, Japan (Wakeman et al., 2015). In addition to dinoflagellates, some euglenophytes including *Euglenaria clavata*, which occurred in summer-autumn at moderate cell abundance in the ECPB, have been found to produce euglenophycin, a toxin associated with ichthyotoxic effects (i.e., fish kills) (Zimba et al., 2017).

Like in other large estuaries [e.g. Chesapeake Bay, USA (Semcheski et al., 2016)], both diversity and abundance of potentially toxic cyanobacteria in the ECPB were much higher than that of dinoflagellates and euglenophytes. Most cyanobacterial genera found in this study (*Anabaena*, *Calothrix*, *Chroococcus*, *Coelosphaerium*, *Lyngbya*, *Merismopedia*, *Oscillatoria* and *Phormidium*) are known to produce compounds with hepatotoxic activity (cyanotoxins), being microcystins the most common of them (Carmichael, 2001; Pearson et al.,



2010). In addition, some of these brackish-water cyanobacterial genera can produce other potent neurotoxins, including anatoxins (from *Anabaena*, *Oscillatoria* and *Phormidium*), saxitoxins (*Anabaena* and *Lyngbya*) and antillatoxin (*Lyngbya*) (Pearson et al., 2010). A group of estuarine species (e.g. *Lyngbya* and *Oscillatoria* spp.) is also capable of producing dermatotoxins such as lyngbyatoxins and aplysiatoxins. Recently, the toxin b-N-methylamino-L-alanine (BMAA) and different novel cytotoxic and apoptosis-inducing compounds have been described from several marine cyanobacteria, including benthic species (Monteiro et al., 2017). Exposure to BMAA has been associated with an increased risk of neurodegenerative diseases such as amyotrophic lateral sclerosis, Parkinson's and Alzheimer's diseases (Bradley et al., 2013). Finally, benthic cyanobacteria belonging to the genera *Hydrocoleum*, *Phormidium*, *Oscillatoria* and *Anabaena* have been linked to acute food poisoning with similar symptoms to those characteristic of ciguatera in New Caledonia, French Polynesia, and Vanuatu, Pacific Ocean (Laurent et al., 2012). Benthic-feeding organisms may be thus exposed to a variety of toxic compounds in the ECPB as cyanobacteria were not only diverse, but also frequent and sometimes very abundant over the investigated period.

The mat-forming *L. cf. aestuarii* was extremely abundant during February, the warmest month, attaining  $5.0 \times 10^4$  cells  $\text{cm}^{-2}$  on the sediments and  $1.4 \times 10^6$  cells  $\text{g}^{-1}$  on macrophytes. Even though this marine/estuarine cyanobacterium is not known to produce the potent lyngbyatoxins, other cytotoxic compounds have been isolated from this species (McIntosh et al., 2013). In fact, the present study reported, additionally, acute toxicity to nauplii of the brine shrimp *A. salina* elicited by an aqueous extract from *L. cf. aestuarii*-dominated mats, as previously reported for strains of other estuarine, benthic filamentous cyanobacteria genera such as *Leptolyngbya*, *Microcoleus*, *Nodularia*, *Nostoc* and *Phormidium* (Lopes et al., 2010). However, low abundances of other potentially toxic cyanobacteria such as *Anabaena* sp., *Calothrix crustacea*, *Phormidium* spp., *Oscillatoria nigroviridis* and the toxic dinoflagellate *P. cf. lima* were found associated with these mats in our study, so that the observed toxicity may not be attributed to

*L. cf. aestuarii* per se, but rather to a toxic benthic microbial consortium. On a different perspective, bioactive compounds from benthic microalgae may possess potential therapeutic activity against pathogenic agents (e.g. viruses, bacteria, fungi) and several diseases including cancer (Tan, 2010; Zimba et al., 2016; Espiritu et al., 2017).

Besides the benthic microalgae, four species of potentially harmful planktic microalgae were also reported in this study occurring sporadically on the sampled substrates: the dinoflagellates *Prorocentrum minimum* and *Dinophysis cf. acuminata*, the diatom *Pseudo-nitzschia* sp. and the cyanobacterium *Coelosphaerium* sp. Most *P. minimum* strains investigated so far were harmless, but some of them seemed to produce neurotoxic compounds that rapidly killed mice when injected intraperitoneally (Grzebyk et al., 1997), and exhibited acute toxicity to mollusks larvae and *Artemia* nauplii (Wickfors and Smolowitz, 1995). Similarly, among several strains of *Pseudo-nitzschia* spp. isolated from southern Brazil, including in the ECPB area, the amnesic toxin domoic acid was only detected at low levels in two species, *P. calliantha* and *P. cf. multiseriis* (Wadt et al., 2017). Moreover, both *P. minimum* and *Pseudo-nitzschia* sp. were found at very low cell densities and only within the MPB collected in one or two out of four sampled dates. Finally, *D. cf. acuminata*, species complex responsible for the vast majority of DSP cases worldwide (see Kamiyama and Suzuki, 2009; Reguera et al., 2012), only appeared at one occasion and at very low cell abundances in our benthic samples. In contrast, *Dinophysis* spp. can be frequently found in plankton samples (Mafra et al., 2006, 2014) and is commonly associated with the contamination of filter-feeding organisms in the outer sector of the ECPB and adjacent continental shelf (Mafra et al., 2014, 2019). Therefore, transfer of toxic *Dinophysis* cells - and their toxins - from the water column to the benthos can be eventually much more significant than that reported during the present investigation. Indeed, only two months after the sampling period covered by this study, transfer of OA to benthic-feeders (e.g. bivalve mollusks, the ghost shrimp *Callinectes major* and the sand dollar *Mellita quinquesperforata*) has been reported during a massive *D. cf. acuminata* bloom in this region (Mafra et al., 2019).

## CONCLUSION

This work confirms the importance of benthic microalgae as primary producers in mud-sand flats of this large, tide-driven estuarine system, where mangrove and phytoplankton producers have received much more attention in past studies on ecosystem ecology. Moreover, our investigation has indicated that, at certain occasions, benthic algae are not only important sources of energy but also of toxic compounds to benthic food webs in this area. Potentially toxic microalgae were represented within different MPB taxonomic groups (cyanobacteria, dinoflagellates, euglenophytes and diatoms) in this subtropical estuarine system. Despite the dominance of non-toxic diatoms in terms of richness and abundance, the mat-forming *Lyngbya* cf. *aestuarii* and other dominant cyanobacteria were very abundant in February, during austral summer, strongly associated with higher rainfall. Contrarily, dinoflagellates, including the toxic species *Amphidinium operculatum* and *Prorocentrum* cf. *lima*, were relatively dominant in drier months characterized by lower water turbulence (July and September). These findings are very relevant for artisanal fisheries and aquaculture - important regional economical activities and a food security issue in this low-income region - as well as for conservation actions towards key species that use this ecosystem as nursery and feeding area, such as mollusks, crustaceans, fish and the endangered sea turtle *Chelonia mydas*. The occurrence of toxic benthic microalgal species in the ECPB, which had been vastly underestimated so far, should be therefore considered in future studies and actions addressing the risks for environmental and human health in this region.

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## AUTHOR CONTRIBUTIONS

A.R.M.-G.: Investigation; Methodology; Visualization; Writing – original draft; Writing – review & editing.  
M.C.B.: Formal Analysis; Writing – review & editing.  
L.L.M.Jr.: Conceptualization; Funding Acquisition; Project Administration; Resources; Supervision; Writing – review & editing.

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## SUPPLEMENTARY MATERIAL

**Appendix A.** List of benthic microalgae found on different substrates collected at three stations along the ECPB, their mean and maximum (between brackets) abundance [cells cm<sup>-2</sup> for sediment (S) and cells g<sup>-1</sup> wet weight for macrophyte (M)], and the relative abundance (RA) values (%). Asterisks indicate potentially toxic species.

|  |   | July 2015       |                      | Sept. 2015      |                      | February 2016  |                      | May 2016       |                      |
|--|---|-----------------|----------------------|-----------------|----------------------|----------------|----------------------|----------------|----------------------|
|  |   | Mean<br>(Max.)  | RA (%)               | Mean<br>(Max.)  | RA (%)               | Mean<br>(Max.) | RA (%)               | Mean<br>(Max.) | RA (%)               |
| <b>EUCARIOTA</b>   |   |                 |                      |                 |                      |                |                      |                |                      |
| <b>Dinoflagellates</b>   |   |                 |                      |                 |                      |                |                      |                |                      |
| <i>Amphidiniopsis</i> sp.<br>Woloszynska   | S | 98.7<br>(750)   | 0.431                |                 |                      |                |                      | 87.5<br>(503)  | 0.122                |
|  | M | 0.513<br>(13.0) | 1.1×10 <sup>-4</sup> | 63.1<br>(780)   | 0.024                |                |                      |                |                      |
| <i>Amphidinium operculatum</i><br>Claparède and<br>Lachmann* 1,2                               | S | 3.72<br>(26.5)  | 0.023                | 4.42<br>(26.5)  | 0.031                | 47.1<br>(199)  | 9.2×10 <sup>-3</sup> |                |                      |
|  | M | 91.2<br>(1370)  | 0.023                | 197<br>(2160)   | 0.061                | 71.1<br>(1110) | 4.7×10 <sup>-3</sup> | 19.6<br>(153)  | 2.3×10 <sup>-3</sup> |
| <i>Amphidinium bipes</i><br>Herdman  | S | 34.6<br>(159)   | 0.153                |                 |                      | 7.81<br>(33.0) | 1.5×10 <sup>-3</sup> |                |                      |
|  | M | 0.632<br>(12.7) | 1.3×10 <sup>-4</sup> | 33.4<br>(433)   | 9.5×10 <sup>-3</sup> | 64.8<br>(948)  | 4.3×10 <sup>-3</sup> |                |                      |
| <i>Amphidinium</i> sp.<br>E Claparède and L<br>Lachmann* 1,2                                   | S |                 |                      |                 |                      | 11.7<br>(132)  | 2.3×10 <sup>-3</sup> |                |                      |
|  | M |                 |                      | 493<br>(2880)   | 0.142                |                |                      |                |                      |
| <i>Ankistrodinium<br/>semilunatum</i> (Herdman)<br>Hoppenrath, Murray,<br>Sparmann and Leander | S | 518<br>(2250)   | 2.26                 |                 |                      |                |                      | 83.8<br>(265)  | 0.122                |
|  | M | 1.83<br>(43.3)  | 3.8×10 <sup>-4</sup> |                 |                      |                |                      |                |                      |
| <i>Coolia malayensis</i> Leaw, P-T<br>Lim and Usup* 3  | S |                 |                      |                 |                      |                |                      |                |                      |
|  | M |                 |                      | 7.22<br>(130)   | 2.1×10 <sup>-3</sup> |                |                      |                |                      |
| <i>Ceratium fusus</i> (Ehrenberg)<br>Dujardin  | S |                 |                      |                 |                      |                |                      |                |                      |
|  | M | 0.483<br>(13.0) | 2.5×10 <sup>-5</sup> |                 |                      |                |                      |                |                      |
| <i>Dinophysis</i> cf. <i>acuminata</i><br>Claparède and Lachmann* 1                            | S |                 |                      |                 |                      |                |                      |                |                      |
|  | M | 0.041<br>(0.97) | 7.4×10 <sup>-6</sup> |                 |                      |                |                      |                |                      |
| <i>Gymnodinium</i> sp. F Stein   | S | 1.53<br>(26.5)  | 6.4×10 <sup>-3</sup> |                 |                      |                |                      |                |                      |
|  | M | 3.71<br>(73.3)  | 7.9×10 <sup>-4</sup> | 5.23<br>(74.3)  | 1.5×10 <sup>-3</sup> |                |                      |                |                      |
| <i>Heterocapsa</i> sp. F Stein   | S | 2.94<br>(26.5)  | 0.013                |                 |                      |                |                      |                |                      |
|  | M | 168<br>(2930)   | 0.0431               | 0.342<br>(7.42) | 9.6×10 <sup>-5</sup> |                |                      |                |                      |
| <i>Peridinium quinquecorne</i><br>Abé  | S | 42.3<br>(583)   | 0.191                |                 |                      | 21.4<br>(166)  | 4.2×10 <sup>-3</sup> |                |                      |
|  | M | 4.74<br>(86.6)  | 9.9×10 <sup>-4</sup> | 11.8<br>(282)   | 3.3×10 <sup>-3</sup> | 36.4<br>(553)  | 2.4×10 <sup>-3</sup> |                |                      |

|  |   |                                   |                      |                      |                      |                                   |                      |                                   |                      |
|--|---|-----------------------------------|----------------------|----------------------|----------------------|-----------------------------------|----------------------|-----------------------------------|----------------------|
| <i>Prorocentrum cf. fukuyoi</i> S<br>Murray and Y Nagahama           | S | 7.34<br>(53.0)                    | 0.033                | 10.3<br>(53.0)       | 0.061                | 144<br>(530)                      | 0.033                | 102<br>(344)                      | 0.142                |
|  | M | 11.0<br>(155)                     | $2.3 \times 10^{-3}$ | 123<br>(1030)        | 0.042                | 145<br>(715)                      | $9.6 \times 10^{-3}$ | 248<br>(86.6)                     | 0.032                |
| <i>Prorocentrum minimum</i><br>(Pavillard) J. Schiller* <sup>1</sup> | S |                                   |                      |                      |                      | 19.5<br>(331)                     | $3.8 \times 10^{-3}$ |                                   |                      |
|  | M | 6.53<br>(89.6)                    | $1.4 \times 10^{-3}$ |                      |                      | 1.54<br>(54.1)                    | $9.9 \times 10^{-5}$ |                                   |                      |
| <i>Prorocentrum lima</i> (Ehrenb.)<br>Dodge* <sup>1</sup>            | S | 57.5<br>(361)                     | 0.253                | 118<br>(331)         | 0.672                | 58.7<br>(199)                     | 0.013                | 7.44<br>(26.8)                    | 0.012                |
|  | M | 192<br>(4024)                     | 0.043                | 78.3<br>(513)        | 0.022                | 267<br>(2410)                     | 0.022                | 14.0<br>(86.6)                    | $1.7 \times 10^{-3}$ |
| <i>Prorocentrum micans</i><br>Ehrenberg                              | S |                                   |                      |                      |                      |                                   |                      |                                   |                      |
|  | M | 0.293<br>(10.4)                   | $6.1 \times 10^{-5}$ | 10.1<br>(128)        | $6.7 \times 10^{-4}$ |                                   |                      |                                   |                      |
| <i>Prorocentrum scutellum</i><br>Schröder                            | S | 2.92<br>(53.0)                    | 0.012                | 1.53<br>(26.5)       | $8.3 \times 10^{-3}$ |                                   |                      | 3.74<br>(26.5)                    | $5.2 \times 10^{-3}$ |
|  | M | 0.122<br>(4.33)                   | $2.6 \times 10^{-5}$ | 2.73<br>(65.0)       | $7.7 \times 10^{-4}$ | 14.3<br>(128)                     | $9.4 \times 10^{-4}$ |                                   |                      |
| Prorocentrum sp.<br>Ehrenberg* <sup>4</sup>                          | S | 21.1<br>(301)                     | 0.091                | 5.94<br>(106)        | 0.033                | 30.2<br>(265)                     | $5.9 \times 10^{-3}$ | 8.82<br>(53.0)                    | 0.013                |
|  | M | 19.6<br>(130)                     | $4.1 \times 10^{-3}$ | 77.0<br>(601)        | 0.024                | 13.4<br>(127)                     | $8.8 \times 10^{-4}$ | 10.4<br>(81.2)                    | $1.2 \times 10^{-3}$ |
| <i>Pyrocystis</i> sp. Wyville-<br>Thompson                           | S |                                   |                      | 7.31<br>(132)        | 0.044                |                                   |                      |                                   |                      |
|  | M | 0.122<br>(4.33)                   | $2.5 \times 10^{-5}$ |                      |                      |                                   |                      |                                   |                      |
| <i>Sinophysis</i> sp. D Nie and<br>C-C Wang                          | S | 36.3<br>(516)                     | 0.165                |                      |                      | 5.83<br>(33)                      | $1.1 \times 10^{-3}$ |                                   |                      |
|  | M | 1.43<br>(48.9)                    | $2.9 \times 10^{-4}$ | 1.4<br>(37.1)        | $4.1 \times 10^{-4}$ | 11.0<br>(322)                     | $7.2 \times 10^{-4}$ |                                   |                      |
| <i>Togula jolla</i> F Jørgensen, S<br>Murray and N Daugbjerg         | S |                                   |                      |                      |                      |                                   |                      | 0.123<br>(4.34)                   | $2.5 \times 10^{-5}$ |
|  | M |                                   |                      |                      |                      |                                   |                      |                                   |                      |
| <b>Diatoms</b>   |   |                                   |                      |                      |                      |                                   |                      |                                   |                      |
| <i>Pseudo-nitzschia</i> sp. H<br>Peragallo* <sup>5,6</sup>           | S | 75.0<br>(1350)                    | 0.332                |                      |                      |                                   |                      |                                   |                      |
|  | M | 1.44<br>(52)                      | $3.1 \times 10^{-4}$ |                      |                      |                                   |                      |                                   |                      |
| Pennate diatoms  | S | 1730<br>(7290)                    | 7.5                  | 16,400<br>(36,700)   | 93.0                 | 449,100<br>(1.2×10 <sup>6</sup> ) | 87.7                 | 39,800<br>(71,500)                | 56.4                 |
|  | M | 580,900<br>(3.4×10 <sup>6</sup> ) | 95.1                 | 260,600<br>(572,000) | 74.2                 | 11,900<br>(1.3×10 <sup>6</sup> )  | 83.6                 | 810,000<br>(5.6×10 <sup>6</sup> ) | 96.6                 |
| Centric diatoms  | S | 20,000<br>(74,100)                | 87.5                 | 1050<br>(3840)       | 5.95                 | 18,700<br>(30,500)                | 3.71                 | 27,700<br>(92,700)                | 3.92                 |
|  | M | 23,100<br>(445,700)               | 4.64                 | 85,700<br>(234,000)  | 24.4                 | 16,400<br>(97,500)                | 1.12                 | 15,200<br>(71,500)                | 1.83                 |

| <b>Euglenophytes</b>   |   |                 |                      |                  |                      |                                   |                      |                  |       |
|--|---|-----------------|----------------------|------------------|----------------------|-----------------------------------|----------------------|------------------|-------|
| <i>Euglenaformis proxima</i><br>(Dangeard) Bennett &<br>Triemer                                | S | 0.742<br>(13.2) | $3.2 \times 10^{-3}$ | 1.54<br>(26.5)   | $8.3 \times 10^{-3}$ | 3.92<br>(66.2)                    | $7.6 \times 10^{-4}$ |                  |       |
|  | M | 574<br>(16,500) | 0.122                | 505<br>(7200)    | 0.142                | 39.9<br>(553)                     | $2.6 \times 10^{-3}$ | 134<br>(1060)    | 0.022 |
| <i>Euglenaria clavata</i> (Skuja)<br>Karnkowska and EW<br>Linton* <sup>6</sup>                 | S | 0.742<br>(13.2) | $3.2 \times 10^{-3}$ |                  |                      | 138<br>(1320)                     | 0.033                | 1850<br>(5560)   | 2.64  |
|  | M |                 |                      | 0.453<br>(16.2)  | $1.3 \times 10^{-4}$ |                                   |                      | 7290<br>(71,500) | 0.923 |
| <b>Silicoflagellates</b>   |   |                 |                      |                  |                      |                                   |                      |                  |       |
| <i>Dictyocha speculum</i><br>Ehrenberg   | S |                 |                      | 30.3<br>(132)    | 0.174                | 1.92<br>(33.1)                    | $3.8 \times 10^{-4}$ |                  |       |
|  | M | 2.02<br>(44.8)  | $4.2 \times 10^{-4}$ | 22.0<br>(171)    | $6.2 \times 10^{-3}$ |                                   |                      |                  |       |
| <i>Dictyocha octonaria</i><br>Ehrenberg  | S |                 |                      |                  |                      |                                   |                      |                  |       |
|  | M |                 |                      | 10.8<br>(130)    | $3.1 \times 10^{-3}$ |                                   |                      |                  |       |
| <b>EUBACTERIA<br/>Cyanobacteria</b>  |   |                 |                      |                  |                      |                                   |                      |                  |       |
| <i>Anabaena</i> sp. ex Bornet<br>and Flahault* <sup>7,9</sup>                                  | S |                 |                      |                  |                      | 10,500<br>(53,000)                | 2.03                 |                  |       |
|  | M | 2.32<br>(84.4)  | $5.0 \times 10^{-4}$ |                  |                      | 22,300<br>(218,300)               | 1.53                 |                  |       |
| <i>Calothrix crustacea</i><br>C.Agardh ex Bornet and<br>Flahault* <sup>7,9</sup>               | S |                 |                      |                  |                      |                                   |                      | 585<br>(3180)    |       |
|  | M |                 |                      | 2640<br>(24,100) | 0.752                |                                   |                      | 423<br>(3810)    | 0.052 |
| <i>Chroococcus</i> sp. Nägeli* <sup>7</sup>  | S | 1.52<br>(26.5)  | $6.4 \times 10^{-3}$ |                  |                      | 146<br>(530)                      | 0.032                | 89.8<br>(318)    | 0.134 |
|  | M | 33.7<br>(898)   | $7.1 \times 10^{-3}$ | 40.0<br>(720)    | 0.014                | 1150<br>(9530)                    | 0.083                | 178<br>(1300)    | 0.024 |
| <i>Coelosphaerium</i> sp. Nägeli* <sup>7</sup>   | S | 1.52<br>(26.5)  | $6.4 \times 10^{-3}$ |                  |                      |                                   |                      |                  |       |
|  | M | 6.45<br>(224)   | $1.3 \times 10^{-3}$ | 50.1<br>(1800)   | 0.015                | 273<br>(2060)                     | 0.024                |                  |       |
| <i>Lyngbya</i> cf. <i>aestuarii</i><br>(Mertens in Jürgens)<br>Liebman ex Gomont* <sup>8</sup> | S | 21.6<br>(390)   | 0.093                |                  |                      | 5870<br>(50,300)                  | 1.14                 |                  |       |
|  | M | 5.74<br>(104)   | $1.2 \times 10^{-3}$ |                  |                      | 201,300<br>( $1.45 \times 10^6$ ) | 13.3                 | 5120<br>(38,350) | 0.614 |
| <i>Merismopedia elegans</i> * <sup>7,9</sup>   | S | 1.53<br>(13.2)  | $0.4 \times 10^{-3}$ |                  |                      | 5800<br>(50,300)                  | 1.1                  |                  |       |
|  | M |                 |                      | 60.0<br>(891)    | 0.023                | 3450<br>(107,300)                 | 0.233                | 272<br>(2000)    | 0.034 |
| <i>Oscillatoria nigroviridis</i><br>Thwaites ex Gomont* <sup>7,9</sup>                         | S |                 |                      |                  |                      |                                   |                      |                  |       |
|  | M |                 |                      |                  |                      | 1070<br>(7023)                    | 0.11                 |                  |       |
| <i>Phormidium</i> sp. 1 Kützing<br>ex Gomont* <sup>7,9</sup>                                   | S | 210<br>(1980)   | 0.921                |                  |                      | 2000<br>(165,800)                 | 3.92                 | 223<br>(1340)    | 0.334 |
|  | M | 38.5<br>(507)   | $7.9 \times 10^{-3}$ | 639<br>(5410)    | 0.182                | 129<br>(2330)                     | $8.2 \times 10^{-3}$ |                  |       |

|  |   |                |                      |                   |                      |
|--|---|----------------|----------------------|-------------------|----------------------|
| <i>Phormidium</i> sp. 2 Kützing<br>ex Gomont <sup>*7,9</sup> | S |                |                      | 1600<br>(10,600)  | 0.313                |
|  | M | 4.33<br>(86.6) | $1.2 \times 10^{-3}$ | 3630<br>(107,300) | 0.242                |
| <i>Spirulina</i> sp. ex Gomont                               | S |                |                      |                   |                      |
|  | M |                |                      | 83.6<br>(3010)    | $5.5 \times 10^{-3}$ |

References: <sup>1</sup> Moestrup et al., 2009; <sup>2</sup> Yasumoto et al., 1987; <sup>3</sup> Wakeman et al., 2015; <sup>4</sup> Moreira-González et al., 2019; <sup>5</sup> Trainer et al., 2000; <sup>6</sup> Zimba et al., 2017; <sup>7</sup> Carmichael, 2001; <sup>8</sup> McIntosh et al., 2013; <sup>9</sup> Lopes and Vasconcelos, 2011.