**Phytoplankton communities in a turbid, acidified tropical estuary (Brunei, Borneo, South East Asia)**

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**Abstract:** Characterization of phytoplankton communities is essential to understanding the ecological functioning of pelagic marine systems. Despite this, our knowledge of phytoplankton communities remains inadequate for many tropical habitats, including estuaries. Tropical estuaries are often highly turbid, have a low buffering capacity and may experience acidification, naturally through microbial degradation and run-off from acid sulphate soils (ASS), or various anthropogenic causes. Here, we describe phytoplankton communities from the turbid, acidified, and eutrophic Sungai Brunei and Brunei Bay estuarine system (Borneo, South East Asia). Four sampling stations were selected, representing the full spectrum of the salinity (0.4 - 28.5 PSU) and pH (5.87 - 8.06) gradient associated with this system. A total of 26 microalgal families of phytoplankton (22 genera of diatoms, seven of dinoflagellates, and one of ciliates) were recorded in the survey, which was carried out over one year. Phytoplankton density ranged from 7 to 9107 cells ml-1. Diatoms were a dominant component of the communities, with *Nitzschia* spp., *Rhizosolenia* spp., and *Leptocylindrus* sp. reaching the highest abundances. Salinity, pH and dissolved oxygen (DO) were positively correlated with the plankton abundances, and all typically declined landwards. Phytoplankton communities were strongly influenced by the effect of season (explaining 30% of the total variance in phytoplankton data) and sampling site (20% of total variation). The interactive effects of pH and salinity, and of pH and temperature, explained 16.7% and 17.5% of the total observed variation, respectively. This study presents the first baseline information showing the response of a tropical estuarine phytoplankton community to acidification.

**Keywords:** acidification, Brunei, pH, phytoplankton, tropical estuary, turbid

**Introduction**

Marine phytoplankton contribute to up to half of global primary production, providing organic matter for the great majority of marine life and being crucial to the global carbon cycle (Longhurst et al., 1995; Cassar et al., 2003; Falkowski, 2012). Estuarine phytoplankton production amounts to 256 g C m-2 per year, which places estuarine systems among the most productive globally (Boynton et al., 1982; Costanza et al., 1997). Productivity and biodiversity are not automatically connected, and high biodiversity does not seem to be an *a priori* condition required for successful functioning of estuarine ecosystems (Elliot and Quintino, 2007). As with other transitional waters, and transitional zones in general, estuaries provide highly variable, naturally stressed habitats that are also commonly exposed to anthropogenic disturbance (de Jonge et al., 2002; Pradhan et al., 2009; Hu and Cai, 2013; Nirmal Kumar et al., 2013). Indeed, estuaries are considered to be amongst the most threatened biological systems, and it is also expected that negative impacts of the many anthropogenic perturbations will be more severe in tropical than temperate regions (Downing et al., 1999; Su et al., 2004).

 Although estuaries have long been considered living “macro-laboratories” for studying the effects of various stressors (e.g. highly variable salinity, temperature, turbidity, high organic matter and nutrient concentrations, heavy metals) the majority of studies to date have focused on benthic organisms (Hopkinson et al., 1999; Morrisey et al., 2003; Alfaro, 2006; Widdicombe and Spicer, 2008; Miller et al., 2009; Green and Barnes, 2010; Majewska et al., 2012; Hossain and Marshall, 2014). In contrast, knowledge of estuarine phytoplankton communities is poor and largely incomplete, especially in tropical regions.

 Given heightened concerns about the ecological consequences of increase in global atmospheric CO2 concentration, it is remarkable that to date little consideration has been given to estuarine acidification and ecological responses thereto. Various hydrographic surveys, time series data and models for open seas indicate clearly that absorption of anthropogenic CO2 across the ocean surface is, and will be, followed by decrease of seawater pH (Caldeira and Wickett, 2003; Orr et al., 2005; Widdicombe and Spicer, 2008; Fabry et al., 2008; Martin et al., 2008; Duarte et al., 2013). In contrast, the carbonate system of many estuaries is characterized by supersaturated water pCO2 levels at highest reaches, derived from natural heterotrophic microbial decomposition, such that these estuaries are sources rather than sinks for atmospheric CO2 (Raymond et al., 2000; Sarma et al., 2001; Thottathil et al., 2008). Additionally, in many regions across the globe, particular geological formations result in acid (iron) sulphate soils, and consequently in highly acidic freshwater runoff from these soils (Cook et al., 2000; Marshall et al., 2008; Grealish and Fitzpatrick, 2013). Dissociation products of strong acids (HNO3 and H2SO4) are also derived from from industry and agriculture and further contribute to acidification. Although, on a global scale, the contribution of such acids to anthropogenic ocean acidification may be minor, they can be far more significant in coastal waters and estuaries. and impact local fisheries, industries, coastal centres and communities (Doney et al., 2009; Feely et al., 2010; Hu and Cai, 2013). Acidification in estuaries is further aggravated by low salinity and, hence, poor buffering capabilities (Miller et al., 2009). Futhermore, recent studies have linked acidification to natural and artificial eutrophication through raising primary production (Cai et al., 2011; Sunda and Cai, 2012; Wallace et al., 2014). It is, thus, clear that estuarine acidification is multifactorial and can be an important ecological phenomenon, deserving more research consideration, especially to improve conservation and management efforts in estuarine systems.

Although various investigations have revealed some important aspects of the responses of marine biota to lowered pH, these have generally focused on shell-forming calcifying organisms, as these organisms are likely to experience increased shell dissolution (Dove and Sammut, 2007; Marshall et al., 2008; Miller et al., 2009; Green and Barnes, 2010; Waldbusser et al., 2011; Gazeau et al., 2013; Hossain and Marshall, 2014). A number of studies have attempted to assess acidification effects on phytoplankton under controlled laboratory conditions (e.g. Hinga, 2002; Hansen, 2002; Low-Décarie et al., 2011; Brading et al., 2011; Lohbeck et al., 2012), but data on *in situ* microalgal responses to lowered pH are scarce (e.g. Geelen and Leuven, 1986). Acidification gradients in open waters are uncommon, and studies on naturally occurring CO2-driven pH gradients, such as those associated with the volcanic vents, almost exclusively describe benthic communities (Hall-Spencer et al., 2008; Turnicliffe et al., 2009; Johnson et al., 2012; Porzio et al. 2013). The most comprehensive recent work on acid sulphate estuaries has been carried out in the temperate region of Sydney, Australia (Amaral et al., 2011a,b, 2012a,b)

The Brunei River estuary – an eutrophic, acidified, highly turbid aquatic system – offers an opportunity to investigate tropical phytoplankton communities along a relatively steep gradient of salinity and pH. In this work, the first to study the phytoplankton of an estuarine system in this region, we aimed to characterize the spatial and temporal patterns in microalgal communities, and assess potential effects of variable environmental conditions on their densities and composition.

**Materials and Methods**

*Sampling area*

The Brunei River estuary system occupies an area of ca. 1380 km2 of Brunei Bay, including the Inner Brunei Bay. Three main rivers (Sungai Limbang, Sungai Temburong, and Sungai Brunei) have a major freshwater input into the estuary. The area is divided into Brunei Channel and Temburong Channel (Currie, 1979). Along most of the banks, the system is fringed with extensive *Rhizophora* mangrove stands (Chua et al., 2014). Due to the equatorial tropical climate the region is characterized by seasonal heavy rainfalls and high temperatures throughout the year. The local tides are diurnal or semi-diurnal, with maximum daily amplitudes of around 2 m in the vicinity of the estuary’s mouths (Hossain and Marshall, 2014; Hossain et al., 2014). Brunei’s capital and largest city, Bandar Seri Begawan (BSB; population = 200,000) is located on the banks of the Sungai Brunei estuary. A large fraction of the city’s domestic waste as well as treated and untreated sewage effluent is discharged into the estuary (Yau, 1991; Marshall et al., 2008). Furthermore, a large traditional water village (Kampong Ayer, current population c. 15,000) is located on the estuarine water near BSB. The Sungai Brunei estuary is distinctly brown and highly turbid, and receives a significant organic load from both mangroves and urban centres. In addition, the system is affected by eutrophication, acidic sulphate groundwater inflows, and heterotrophic metabolism. As a result, steep pH and salinity gradients extend along the estuary. Due to local dynamics, both pH and salinity are highly variable and range between 4.0-8.0 and 0-34 PSU, respectively (Bolhuis et al., 2014).

*Phytoplankton sampling*

Phytoplankton samples were collected at four stations located along the Brunei River estuary, Chermin Island (S1; 4.927650°N, 115.020406°E), Sungai Bunga (S2; 4.900956°N, 114.998353°E), Pintu Malim (S3; 4.888897°N, 114.979133°E), and Kedayan River-Kiulap (S4; 4.886733°N, 114.936767°E). These were selected to display a strong gradient of pH and salinity (Fig. 1). Sampling was carried out every between August 2011 and June 2012. Heavy rainfall associated with the Asian monsoons (north-eastern monsoon, NEM, from November to March; south-western monsoon, SWM, from May to September), as well as the dry inter-monsoon periods (April and October), significantly affect the local conditions (Adam A., personal observations).

Phytoplankton samples were collected by towing a plankton net (20 µm mesh) behind a small boat ca. 0.5 m under the water surface. On each sampling occasion, a similar procedure was carried out for 3 min at a constant speed (2 ms-1). Subsequently, collected material was concentrated, transferred into 100 ml containers, and preserved with Lugol’s iodine solution. All sampling took place during the daytime, at the same tidal level (just after high tide). Tidal levels were estimated using TideComp v.7.04 (Pangolin, Bristol, UK) software.

Sedgewick-Rafter counting slides were used for phytoplankton identification and quantification. Prior to observation the samples were homogenised by gently agitating the sample bottle. From a well-mixed sample, 1 ml was dispensed into the counting cell and viewed under a compound microscope at 40x and 100x magnifications. This procedure was repeated three times and the final result was expressed as the mean value of the three counts. Collected phytoplankton were identified to the lowest taxonomic level possible using relevant references (e.g. Ehrenberg, 1844; Lauder, 1864; Schmidt, 1874, 1878, 1888, 1890, 1892, 1893; Shirota, 1966; Tomas, 1997). Scanning electron microscopy of phytoplankton samples was undertaken in Italy (II University of Naples). Samples were shipped in Lugol’s solution. In order to remove all organic matter, material was digested following a slight modification of the method of von Stosch (Hasle and Syvertsen, 1997) using a mixture of boiling concentrated acid (64% nitric acid and 97% sulphuric acid added at a 1:3 volume ratio). Following digestion and centrifugation, cleaned material was rinsed and diluted with deionized water. Subsequently, the oxidized suspension was placed on aluminum stubs with a carbon tape on a 3-µm Whatman™ polycarbonate membrane filter. The stubs were sputter-coated with gold-palladium and examined in a ZEISS Supra 40 SEM microscope at 5 kV (Centro Grandi Apparecchiature, II University of Naples, Naples, Italy).

*Physicochemical properties of water*

Physicochemical properties of water (salinity, temperature and pH) were measured *in situ* (at 0.5 m depth) at the time of sampling using a calibrated pH and salinity meter (Hanna Instruments, USA, two points calibration, used from August 2011 to January 2012; YSI Model 63, Yellow Springs Instrument Co., three points calibration, used from February to June 2012). For dissolved oxygen (DO) measurements, a 1l seawater sample kept in an airtight polyethylene jar was taken to the laboratory and DO level was measured using a NexSens WQ-DO Sensor. The sensor was calibrated in a 100% oxygen saturated environment (air calibration). In order to ensure a proper polarization of the electrodes, the probe was warmed up for 15-30 min before calibration. Barometric pressure (745 mmHg) and salinity were set before the DO reading was taken. Correlation between these environmental parameters (pH, salinity, temperature, and DO) and population density were analysed using SPSS Ver. 15.

*Statistical analyses*

Statistical analyses were performed using the PAST 2.17b (Hammer et al., 2001), PRIMER Ver. 5 (Clark and Warwick, 2001), and Canoco 5 (ter Braak and Šmilauer, 2012) software. Although taxa were identified to the lowest taxonomic level possible, further analyses were performed on generic-level data to avoid detection of false patterns due to potential misidentification. A similarity percentage analysis (SIMPER) was run to identify phytoplankton taxa responsible for the similarity within groups. To evaluate the relationship between the phytoplankton communities and measured environmental variables, a constrained ordination method was used. Prior to this analysis, an unconstrained unimodal ordination (detrended correspondence analysis, DCA) was performed and the length of its ordination axes was measured. On this basis (the longest axis = 2.4 turnover units) the linear method was selected as the most appropriate for the analysed dataset (Šmilauer and Lepš, 2014). Subsequently, redundancy analysis (RDA) and partial RDA were performed on log-transformed abundance data. A Monte Carlo permutation test was used to test the significance of the axes (4999 permutations, p < 0.05). In order to select the best subset of the chosen environmental variables to summarize the variation in phytoplankton composition, interactive forward selection was performed. The conditional and simple effects of individual explanatory variables upon the compositional data were assessed using a variation partitioning procedure (Legendre, 2007)

**Results**

*Phytoplankton composition and densities*

A total of 26 microalgal families were found in the Sungai Brunei estuary area during the period of study, with 22 genera of diatoms, seven of dinoflagellates and one family of ciliates (Table 1). Light and scanning electron micrographs of selected taxa are shown in Figures 2 and 3.

 In terms of phytoplankton abundance, the highest density was recorded at Station 3 (S3; 1251 cells ml-1 ± 2544), followed by Station 2 (S2; 1119 cells ml-1 ± 2197), Station 1 (S1; 437 cells ml-1 ± 744) and lastly Station 4 (S4; 118 cells ml-1 ± 265). Generally, the highest phytoplankton densities (up to 9107 cells ml-1, S3) were observed in August and October (Fig. 4). The differences in phytoplankton abundances observed among the four sampling stations during the study period were significant (Kruskal-Wallis, p < 0.05).

 Comparing the communities, the highest average dissimilarity occurred between those found at S4 and S1 (69.2%), S4 and S2 (68.2%), and S4 and S3 (67.6%) while the most similar were the communities from S1 and S2(59.8%; Table 2). Diatoms constituted the main fraction of the observed microalgal communities, with *Nitzschia* (0-61.5% of the total cell number in the sample; 25.9% on average) being the dominant genus. This was followed by two other high-density genera, *Rhizosolenia* (0-52.4%; 24.1% on average) and *Leptocylindrus* (0-75.5%; 21.9% on average). According to the SIMPER analysis, these taxa together with *Chaetoceros* spp*.*, *Thalassionema* spp., *Dinophysis* spp., and *Pleurosigma* spp. were responsible for more than 50% of the dissimilarity between the sampling stations (for further detail see Supplemental material: Tables I-VI).

*Phytoplankton communities vs. environmental factors*

Values of salinity, pH, and DO generally decreased landward, from Station 1 to Station 4 (Table 3), with the differences between the stations being statistically significant (Kruskal-Wallis, p < 0.05). However, the four sampling sites did not differ significantly in terms of water temperature. All of the environment variables (pH, temperature, salinity, DO) were significantly positively correlated with phytoplankton density (Table 4).

*Differences among seasons*

A partial RDA with supplementary variables was performed to test the effect of season on phytoplankton communities (Fig. 5). Sampling site was used as a covariate and, therefore, its effect on phytoplankton communities was removed from the model. The explained variation accounted for 33.2% of the total variance in phytoplankton compositional data (adjusted explained variation = 30.0%). As confirmed by the Monte Carlo permutation test (p = 0.001) this effect was significant. Most of the algal taxa appeared to be correlated (either positively or negatively) with salinity and pH. The spring inter-monsoon period (April) was clearly the season in which the conditions were the least favourable for phytoplankton community development. Although both NEM and SWM samples created highly heterogeneous overlapping groups, samples collected during the two inter-monsoon periods (April and October) formed two distinct clusters, suggesting a substantial seasonal change in phytoplankton communities (Fig. 6).

*Differences among sites*

A partial RDA was run to test the effect of sampling site on the algal communities (Fig. 7). The sampling season was used as a covariate, allowing removal of its effect on algal assemblages from the final model. The explained variation accounted for 25.0% of the total variance in phytoplankton compositional data (adjusted explained variation = 20.5%) and the Monte Carlo permutation test (p = 0.001) confirmed significance of the observed effect. All of the phytoplankton taxa responded negatively to the conditions of Station 4. Only the two benthic taxa (*Achnanthes* and *Navicula*) were more common in samples collected at Station 4 than in those collected at the other stations.

Figure 8 presents a classification of the phytoplankton samples into the four groups according to the site from which they were collected. The overlapping clusters illustrate the spatial continuum throughout the sampling area. Nevertheless, samples collected at Station 1 and Station 4 were more floristically heterogeneous than those collected at the other two stations. In addition, samples collected at Station 4, in contrast with the other samples, tended to be placed toward one side of the first axis of the plot. This suggests a relatively high dissimilarity between groups created by S4 samples and those from the three remaining sample locations.

*Influence of water physicochemical properties*

Forward selection of explanatory variables allowed determination of the most important environmental variables affecting phytoplankton communities. The season of sampling along with the sampling site were used as covariates. pH was indicated as the most suitable predictor of the phytoplankton community composition (p = 0.005), followed by temperature (p <0.01) and salinity (p <0.05).

Variation partitioning by partial constrained ordination (RDA) was then performed to quantify the effects (and their overlap) of pH and salinity as well as pH and temperature on the algal communities. Figure 9 summarizes the variation in phytoplankton composition that can be explained by the joint effect of pH and salinity. The overlap of these effects is expected as pH and salinity are positively correlated. The amount of variation explained by the joint effect of the two environmental variables was 16.7% (p =0.001). The sole (partial) effect of pH explained 2.5% (p = 0.02), while the sole effect of salinity accounted for 0.9 % (p > 0.05) of the total variation (see Supplemental material, Figs 9a and 9b for illustration of variation explained by pH or salinity alone).

Similarly, the partitioning procedure indicated that the joint effect of pH and temperature explained 17.5% of the observed variability among samples (p = 0.001; Fig. 10). The partial effects of pH or temperature alone represented 8.1% (p = 0.001) and 1.6% (p > 0.05) of the total variation, respectively (see Supplemental material, Figs 10a and 10b for illustration of variations explained by pH or temperature alone).

 Figures 11 and 12 illustrate the change in the number of phytoplankton taxa recorded in relation to pH, indicating a clear negative correlation with decreasing pH (p = 0.001).

**Discussion**

A total of 22 genera of diatoms, seven of dinoflagellates and one of ciliates were found in the course of the study. The number of taxa found was relatively low compared with some other studies conducted in tropical regions (e.g. Angsupanich and Rakkheaw, 1997; Su et al., 2004; Lueangthuwapranit et al., 2011).

The phytoplankton communities present at the four sampling stations differed significantly in terms of both algal abundance and taxa composition. These differences were not caused by presence or absence of strictly stenohaline taxa, but rather by the dominance of the main common taxa changing along the pH and salinity gradients. The most abundant fractions were constituted by diatoms and dinoflagellates. As noted in several studies (Jacobsen and Andersen, 1994; Muylaert and Sabbe, 1999; Loverde-Oliveira et al., 2009), many of dinoflagellate species may have mixo- or heterotrophic nutrition, which favours their survival in highly turbid conditions. Furthermore, it has been suggested that a higher carbon:chlorophyll ratio in dinoflagellates compared with diatoms may be responsible for their faster growth in conditions of lower pH and elevated CO2 concentrations (Tortell et al., 2008; Low-Décarie et al., 2011). Many diatom species are known to be relatively resistant to a moderate decrease in water pH (up to 0.3 pH units), but their numbers reduce drastically with any further decrease of pH (Geelen and Leuven, 1986, and references therein; Majewska, personal observations). Also, species within some diatom genera (e.g. *Cyclotella*) are known to have clear heterotrophic capabilities, which enable them to survive in low-light conditions (Lylis and Trainer, 1973).

Differences in community composition as well as the relationship between phytoplankton and measured environmental variables were assessed using various statistical analyses. A comparison of several models indicated that the investigated communities were strongly influenced by the effect of season (explaining ca. 30% of the total variance in phytoplankton compositional data) and by the set of factors specifically related to each sampling station (ca. 20% of the total variance).

*pH influence*

Amongst the four environmental variables whose effect was assessed (pH, salinity, temperature, dissolved oxygen), pH appeared to have the strongest influence on phytoplankton assemblages. Although pH is strongly correlated with salinity (Zhou and Rowland, 1997; Hansen, 2002), in many cases its effect when considered alone was opposite to that of salinity, i.e. taxa associated with lower salinity levels appeared to prefer higher pH conditions, and vice versa, when the shared effect of pH and salinity was excluded from the model. A large majority of the taxa had their local optima in stations characterized by relatively high pH values, while the GAM plot confirmed the negative correlation of pH and the number of taxa found in the samples. Other studies on tropical estuarial phytoplankton (Madhu et al., 2007; Krume et al., 2012) have reported similar trends.

*Sampling site influence*

Minima in phytoplankton diversity and abundance were typical for the most landward station, S4, indicating the least favourable conditions for microalgal growth. Although most of the genera could be found in different seasons and in various numbers at all four stations, genera such as *Corethron*, *Cyclotella*, *Guinardia,* and *Triceratium* were never present in samples from S4. A combination of extreme turbidity, resulting in severe light limitation, distinctly lower salinity, pH, and DO values in the proximity of large urban areas (e.g. Kiulap, Gadong, water villages along the estuary) could have affected local phytoplankton markedly more than at the more seaward locations. A relatively high contribution of typically benthic taxa (*Achnanthes* spp., *Navicula* spp.) to the total number of phytoplankton algae found in S4 samples indicated a significant exchange with the benthic communities and a high level of physical disturbance (turbidity, river runoff) that, on the other hand, might also have limited the development of dense phytoplankton assemblages (Muylaert and Sabbe, 1999; Adesalu, 2010; Palleyi et al., 2008).

*Seasonality*

Sampling season proved to have a pronounced effect on the estuarine phytoplankton. The RDA models clearly indicated that the dry inter-monsoon period following NEM heavy rainfalls was the least suitable for the phytoplankton development. The two monsoon seasons resulted in changes in microalgal communities in qualitatively and quantitatively distinct ways. Generally, an increase in phytoplankton abundances was observed directly after the rainfall events. The highest abundances (up to 9107 cells ml-1), however, were associated with the SWM season and not with NEM when a series of the heaviest rainfalls occurred. Wet season and other rainfall events may promote phytoplankton blooms through the leaching of biogenic substances from the adjacent land and the large influx of riverine water highly enriched in organic matter and nutrients (Jackson et al., 1987; Mallin et al., 1993; Angsupanich and Rakkheaw, 1997; Gameiro et al., 2004; Lueangthuwapranit et al., 2011). In addition, algal growth is enhanced by lower tidal variation associated with the monsoon seasons (Palleyi et al., 2008), with tides being recognised as possibly one of the most important factors for local phytoplankton development (Sue et al., 2004; Lueangthuwapranit et al., 2011). In some cases, however, extremely intense rainfall events may negatively affect phytoplankton communities by increasing turbidity (Mallin et al., 1993) and by introducing into the aquatic systems an excessive amount of acid drainage water and heavy metals that are easily leached from the soil in the low pH environment (Russel and Helmke, 2002; Macdonald et al., 2007; Green and Barnes, 2010). Strongly geologically originated ASS are found in the vicinity of the Sungai Brunei estuary (Grealish and Fitzpatrick, 2013; Bolhuis et al., 2014) and the risk of these becoming increasingly damaging to the environment depends greatly on local management and land use practices. Appropriate management practices are not always well understood and it is highly probable that intense leaching during the wet seasons causes a serious threat to water quality and aquatic life (e.g. Dent and Pons, 1995; Sammut et al., 1995). Adam et al. (2011) working in eastern Malaysia and Tan et al. (2006) in the eastern Malacca Straits reported that intense phytoplankton blooms were observed mainly during the NEM. The monsoon influence was associated with strong north-east winds responsible for extensive mixing of the water column. These studies, however, were focused on marine habitats that are only minimally influenced by freshwater and it is unclear how applicable their findings are to the estuarine environment.

Sampling season, site, and water pH explained ca. 60% of the observed variation in the phytoplankton data obtained. However, we recognise that variables not included in these analyses might also significantly affect the communities. This may be inferred from the low eigenvalue of the second axis of the RDA, which was weakly correlated with the selected environmental variables. For instance, high nutrient concentrations often found in the upper part of the estuary (Pintu Malin - Sungai Kedayang), might counter the negative effect of low pH (Jalal et al., 2011). In this region, nutrient levels are likelyto be elevated through sewage treatment activities at Pintu Malim, release of organic materials from Kampong Ayer, small-scale aquaculture facilities, and the effect of Sungai Kedayang inflow, which carries water from a vast urbanized area. Amongst other potentially important influences, grazing and other biological factors (e.g. parasitism) have been suggested to play an important role in phytoplankton dynamics (Canter and Lund, 1953; Muylaert and Sabbe, 1999).

 Although the acute temporal variability in acidification that coincides with seasonal flooding is assumed to relate largely to ASS inflows, finer temporal scale tidal fluctuations in pH during inter-monsoons are more likely to associate with the combination of a lowered buffering capacity, microbial decomposition, and pCO2 saturation in the upper estuarine reaches (Bolhuis et al. 2014; Hossain et al. 2014; Proum unpublished). As many of the processes involved in this complex acidification are natural, with potentially acidic-generating geological formations and biological communities (mangroves) probably dating back to the mid-Miocene, we assume that the phytoplankton taxa living in the Brunei estuary are well adapted to thrive in an environment unsuitable for other planktonic species.

Low-Décarie et al. (2011) considered that changes in community dynamics in environments affected by elevated CO2 concentrations and water acidification are based mostly on physiological differences between major taxa, while the response of species within each taxon is less distinct. If so, this suggests that large shifts in community composition may be predicted to a certain extent without a detailed knowledge of individual species’ ecological preferences. However, most studies underlying such conclusions have been conducted under laboratory conditions, and examining a limited number of taxa (e.g. Richmond et al., 1982; Tortell, 2000; Low-Décarie et al., 2011). It is likely that individual elements of natural phytoplankton communities may behave differently under the influence of the combined effects of multiple environmental factors, and further studies on individual species’ autecology are required to explain phytoplankton dynamics and assess the future changes within microalgal assemblages.

Estuaries are a very specific aquatic environment and the assessment of their potential degradation requires care. Estuarine ecosystems are considered to be naturally stressed due the extremely high variability in parameters such as salinity, pH, temperature, nutrients, dissolved oxygen, sediment dynamics, current speed and direction and light penetration. Nevertheless, while these may be stressful for strictly marine or freshwater organisms, for estuarine-adapted biota they represent the normal state. Indeed, the main characteristics of this natural stress often bear a close resemblance to those of the anthropogenic stressors. Because of that, in the case of estuaries, it is particularly difficult to detect the real effects of the anthropogenic perturbations and one should not rely exclusively on species diversity but rather on the system functional characteristics which are reflected much better by higher taxa composition (Wright, 1982; Elliot and Quintino, 2007). The similarities between highly variable natural stressors and those under anthropogenic influence requires further clarification.

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**Figure captions**

**Fig.1** Sampling stations along Brunei River estuary

**Fig.2** LM and SEM images of selected taxa found in Brunei River estuary; *Pseudonitzschia* sp., *Pleurosigma* sp., *Coscinodiscus* sp., *Bacteriatrum* sp., *Ceratium furca*., *Dinophysis caudata*, *Protoperidinium* sp., …

**Fig.3** Seasonal changes in phytoplankton density from August 2011 to June 2012

**Fig.4** Mean density of the dominant phytoplankton along Brunei River estuary (S1-S3: n=18; S4: n=17) \*p<0.05

**Fig. 5:** Quadplot diagram from RDA summarizing the differences in phytoplankton communities observed over the different sampling seasons. Score scaling is focused on phytoplankton taxa scores (standardized). Eigenvalues: 0.2871, 0.0073, 0.0066

Empty arrows = environmental variables (supplementary)

Solid arrows = phytoplankton taxa

Solid triangles = sampling season: SWM = south-western monsoon, NEM = north-eastern monsoon, A = April (dry inter-monsoon period), O = October (dry inter-monsoon period)

Individual symbols represent dummy variables corresponding to individual levels of a factor (seasons). The distance between the symbols approximates the average dissimilarity of phytoplankton composition between the four sample classes (different seasons) being compared as measured by their Euclidean distance. This approximation is not optimal, because the scaling of scores is not focused on the distances between samples.

“PH” and “Salinity” arrows point in the direction of the steepest increase of environmental variable (suppl.) values. The angle between arrows indicates the correlation between the two variables.

Phytoplankton taxa arrows point in the direction of the steepest increase of the values for corresponding alga. The angle between arrows indicates the correlation between the algae: the approximated correlation is positive when the angle is acute and negative when the angle is larger than 90 degrees. The length of the arrow is a measure of fit for the algal taxon (correlation of that alga with the ordination axes).

**Fig.** **6** Scatter of samples classified into four groups according to the season in which they were collected. The polygons are plotted in the space of the first two RDA axes. Score scaling is focused on phytoplankton taxa scores (standardized).

SWM = south-western monsoon, NEM = north-eastern monsoon, A = April (dry inter-monsoon period), O = October (dry inter-monsoon period)

Black forms represent dummy variables corresponding to individual levels of a factor representing individual groups of samples. The distance between the symbols approximates the average dissimilarity of phytoplankton composition between the sample classes being compared as measured by their Euclidean distance.

The distance between the sample symbols (grey forms) approximates the dissimilarity of their phytoplankton composition (Euclidean distance).

The distance of selected sample symbol (grey forms) from season symbols (black forms) predicts the sample membership in one of the groups. It also reflects the dissimilarity between phytoplankton composition of that sample and average phytoplankton composition of samples belonging to other groups.

**Fig. 7** Biplot diagram from RDA summarizing the differences in phytoplankton communities caused by difference in sampling site. Score scaling is focused on phytoplankton taxa scores (standardized). Only the 15 best fitting taxa are shown (fit into the ordination space on both axes = 6.7%).

Eigenvalues: 0.0732, 0.0135, 0.0054

Solid arrows = phytoplankton taxa

Solid triangles = sampling sites

Triangles represent dummy variables corresponding to individual sampling site. The distance between the symbols approximates the average dissimilarity of phytoplankton composition between the four sampling sites being compared as measured by their Euclidean distance. This approximation is not optimal, because the scaling of scores is not focused on the distances between samples.

Phytoplankton taxa arrows point in the direction of the steepest increase of the values for corresponding alga. The angle between arrows indicates the correlation between the taxa: the approximated correlation is positive when the angle is acute and negative when the angle is larger than 90 degrees. The length of the arrow is a measure of fit for the algal taxon (correlation of that alga with the ordination axes).

**Fig. 8** Scatter of samples classified into four groups according to the sampling site where they were collected. The polygons are plotted in the space of the first two RDA axes. Score scaling is focused on phytoplankton taxa scores (standardized).

White squares represent dummy variables corresponding to individual sampling site representing individual groups of samples. The distance between the symbols approximates the average dissimilarity of phytoplankton composition between the sample classes being compared as measured by their Euclidean distance.

The distance between the sample symbols (grey forms) approximates the dissimilarity of their phytoplankton composition (Euclidean distance).

The distance of selected sample symbol (grey forms) from sampling site symbols (white squares) predicts the sample membership in one of the groups. It also reflects the dissimilarity between phytoplankton composition of that sample and average phytoplankton composition of samples belonging to other groups.

**Figure 9** Biplot diagram from RDA visualizing the joint effect of pH and salinity on phytoplankton communities.Score scaling is focused on alga scores (standardized).Only the 20 best fitting taxa are shown (fit into the ordination space on both axes = 4.2%).

Environmental variable arrows (pH and salinity) point in the direction of the steepest increase of environmental variable values. The angle between arrows indicates the correlation between the two environmental variables.

Phytoplankton taxa arrows point in the direction of the steepest increase of the values for corresponding taxon. The angle between arrows indicates the correlation between the algal taxa: the approximated correlation is positive when the angle is acute and negative when the angle is larger than 90 degrees. The length of the taxa arrows is the multiple correlation of the individual taxon with the ordination axes.

**Supplemental material**

**Figure 9a** Biplot diagram from RDA visualizing the partial effect of pH (after subtraction of the shared effect of pH and salinity) on phytoplankton taxa.

**Figure 9b** Biplot diagram from RDA visualizing the partial effect of salinity (after subtraction of the shared effect of pH and salinity) on phytoplankton taxa.

**Figure 10a** Biplot diagram from RDA visualizing the partial effect of pH (after subtraction of the shared effect of pH and temperature) on phytoplankton taxa.

**Figure 10b** Biplot diagram from RDA visualizing the partial effect of temperature (after subtraction of the shared effect of pH and temperature) on phytoplankton taxa.

**Figure 10** Biplot diagram from RDA visualizing the joint effect of pH and temperature on phytoplankton communities.Score scaling is focused on alga scores (standardized).Environmental variable arrows (pH and temperature) point in the direction of the steepest increase of environmental variable values. The angle between arrows indicates the correlation between the two environmental variables.

Phytoplankton taxa arrows point in the direction of the steepest increase of the values for corresponding taxon. The angle between arrows indicates the correlation between the algal taxa: the approximated correlation is positive when the angle is sharp and negative when the angle is larger than 90 degrees. The length of the taxa arrows is the multiple correlation of the individual taxon with the ordination axes.

**Figure 11** Phytoplankton diversity diagram (RDA). Score scaling is focused on phytoplankton taxa scores (standardized).

Distance between the samples (grey circles) approximates the dissimilarity of their phytoplankton composition as measured by their Euclidean distance. Circle size varies, reflecting the number of taxa within samples: the greater the size, the higher number of taxa found in the sample.

**Figure 12** GAM (generalized additive model) plot of phytoplankton taxa number within samples against pH.

**Table 1** List of the phytoplankton in the Sg. Brunei estuary

|  |  |  |
| --- | --- | --- |
| **CLASS** | **FAMILY** |  |
| **Bacillariophyceae** | Achanthaceae | *Achnanthes*sp |
|  | Amphipleuraceae | *Amphiprora* sp. |
|  | Bacillariaceae | *Nitzschia longissima* (Brébisson) Ralfs |
|  |  | *Nitzschia* spp. |
|  |  | *Pseudo-nitzchia* sp. |
|  | Biddulphiaceae | *Biddulphia mobiliensis* (J.W.Bailey) Grunow |
|  |  | *Biddulphia sinensis* Greville |
|  | Chaetocerotaceae | *Bacteriastrum varians* Lauder |
|  |  | *Bacteriastrum* spp. |
|  |  | *Chaetoceros danicus* Cleve |
|  |  | *Chaetoceros peruvianus* Brightwell |
|  |  | *Chaetoceros* spp. |
|  | Coscinodiscaceae | *Coscinodiscus* spp. |
|  | Hemiaulaceae | *Hemiaulus* sp. |
|  | Leptocylindraceae | *Corethron* sp. |
|  |  | *Leptocylindrus* sp. |
|  | Lithodesmiaceae | *Ditylum sol* Cleve |
|  | Melosiraceae | *Melosira* spp. |
|  | Naviculaceae | *Navicula* spp. |
|  | Pleurosigmataceae | *Pleurosigma* spp. |
|  | Rhizosoleniaceae | *Guinardia striata* (Stolterfoth) Hasle |
|  |  | *Rhizosolenia alata* Brightwell |
|  |  | *Rhizosolenia acuminate* (H.Peragallo) Gran |
|  |  | *Rhizosolenia arafurensis* Castracane |
|  |  | *Rhizosolenia bergonii* H.Peragallo |
|  |  | *Rhizosolenia clevei* Ostenfeld |
|  |  | *Rhizosolenia delicatula* Cleve |
|  |  | *Rhizosolenia pungens*Cleve-Euler |
|  |  | *Rhizosolenia stolterfothii* H.Peragallo |
|  | Stephanodiscaceae | *Cyclotella caspia* Grunow |
|  | Surirellaceae | *Surirella* sp. |
|  | Thalassionemaceae | *Thalassionema frauenfeldii* (Grunow) Tempère&Peragallo |
|  |  | *Thalassionema nitzchioides* (Grunow) Mereschkowsky |
|  |  | *Thalassionema* sp. |
|  | Triceratiaceae | *Triceratium* sp. |
| **Dinophyceae** | Ceritiaceae | *Ceratium furca* (Ehrenberg) Claparède&Lachmann |
|  |  | *Ceratium fusus* (Ehrenberg) Dujardin |
|  |  | *Ceratium trichoceros* (Ehrenberg) Kofoid*Ceratium tripos* (O.F.Müller) Nitzsch |
|  |  | *Ceratium massiliense* (Gourret) Karsten |
|  | Dinophysiaceae | *Dinophysis acuta* Ehrenberg |
|  |  | *Dinophysis caudata* Saville-Kent |
|  | Gonyaulacaceae | *Pyrodinium bahamense* var. *compressum* (Böhm) Steidinger, Tester & F.J.R.Taylor |
|  | Peridiniaceae | *Peridinium* spp. |
|  | Prorocentraceae | *Prorocentrum micans* Ehrenberg |
|  |  | *Prorocentrum granile* Schütt |
|  | Protoperidiniaceae | *Protoperidinium pellucidum* Bergh |
|  |  | *Protoperidinium crassipes* (Kofoid) Balech |
|  |  | *Protoperidinium* sp. |
| **Spirotrichea** | Codonellidae | *Codonella* spp. |
|  |  |  |

**Table 2** Range and mean of the environmental variables

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Station** | **pH** | **Temperature** | **Salinity** | **DO** |
| **1(Pulau Chermin)** | Range | 7.03 – 8.06 | 26.6 – 33.2 | 13.69 – 28.3 | 5.41 – 8.57 |
|  | Mean | 7.58 (±0.59) | 30.06 (±1.63) | 20.29 (±4.29) | 6.74 (±1.84) |
| **2(Sg. Bunga)** | Range | 6.07 – 7.86 | 26.7 – 32.7 | 12.07 – 28.5 | 4.32 – 7.3 |
|  | Mean | 7.25 (±0.45) | 30.02 (±1.45) | 18.72 (±4.88) | 5.69 (±1.14) |
| **3(Pintu Malim)** | Range | 6.72 – 7.80 | 27.2 – 32.2 | 8.34 – 24.4 | 4.23 – 6.92 |
|  | Mean | 7.21 (±0.30) | 30.16 (±1.59) | 16.75 (±4.7) | 5.44 (±0.86) |
| **4(Kedayan River)** | Range | 5.87 – 7.52 | 27.0 – 32.1 | 0.43 – 14.70 | 3.12 – 7.73 |
|  | Mean | 6.58 (±0.42) | 29.39 (±1.32) | 5.98 (±4.5) | 4.94 (±1.24) |

**Table 3** Correlation coefficient between environmental factors and phytoplankton abundance

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **pH** | **Temperature** | **Salinity** | **DO** | **Density** |
| **pH** | 1 |  |  |  |  |
| **Temperature** | 0.490\* | 1 |  |  |  |
| **Salinity** | 0.811\* | 0.514\* | 1 |  |  |
| **DO** | 0.520\* | 0.341\*\* | 0.286\*\* | 1 |  |
| **Density** | **0.496\*** | **0.461\*** | **0. 563\*** | **0.317\*\*** | 1 |

\* p<0.01 \*\*p<0.05

**Fig. 1**



**Fig. 3**

****

**Fig. 4**

 **Abundance (cells/ml) Abundance (cells/ml) Abundance (cells/ml)**

 **Sampling station Sampling station Sampling station**