Prokaryotic abundance, cell size and extracellular enzymatic activity in a human impacted and mangrove dominated tropical estuary (Can Gio, vietnam)

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Running head: Prokaryotic activity in a tropical estuary.

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Keywords: estuary, mangrove, water column, prokaryotic abundance, extracellular enzymatic activity

1. Abstract

Extracellular enzymatic activities constitute the first and limiting step of the whole process of organic matter (OM) cycling in aquatic ecosystems. This study aims to identify the factors controlling prokaryotes ability to hydrolyse OM in an Indo-Pacific tropical mangrove ecosystem (Can Gio, Vietnam). Prokaryotic abundance and leucine-aminopeptidase exo-proteolytic activity (EPA) were measured at vertical (from the sea-surface microlayer to bottom waters), spatial (along a transect within the estuary) and seasonal (wet and dry season) scales. Prokaryotic abundance ranged from 1.2 to 5.7 x 10^9 cells L^{-1} and EPA ranged from 24 to 505 nmol L^{-1} h^{-1} that was relatively similar to other highly productive ecosystems. The estuary was poorly stratified, most probably because of high water turbulence. Yet, exo-proteolytic activity was significantly higher in bottom waters, where higher loads of suspended particulate matter were measured. Seasonal and spatial differences in EPA suggest that the nature of OM transported by the Can Gio mangrove estuary affect EPA. The latter seems to be increased by two “uncommon” situations: the input of fresh and labile OM (*e.g.* shrimp farm effluents) or the lack of labile OM and the need to hydrolyse refractory compounds (*e.g.* during the dry season).

2. Introduction

Prokaryotes and their ability to mineralise organic matter (OM) are essential for controlling the fluxes of carbon and nutrients in coastal waters. Yet, when, where, and how OM is decomposed into nutrients and CO_2 is not fully understood in tropical mangrove ecosystems (Cai 2011). Extracellular enzymatic activities constitute the limiting step of the whole process of OM cycling (Arnosti 2011) and thus, the turnover rates of various compounds have been used to evaluate the efficiency of the microbial community to mineralise OM in coastal waters (Patel et al. 2000, Cunha and Almeida 2006, Bhaskar and Bhosle 2008, Lamy et al. 2009). The leucine-aminopeptidase activity (exo-proteolytic activity, EPA) is one of the most commonly used indicators of OM hydrolysis. It represents the general ability of prokaryotes to hydrolyse proteins (Hoppe 1993) and thus it informs on the efficiency of the ecosystem to recycle nitrogen, which is generally the first limiting nutrient in marine production (Fernandes 2011).

In the present study we explored vertical, spatial and seasonal variations in the prokaryotic compartment of the Can Gio mangrove water column. Prokaryotic abundances, cell sizes and EPA were measured at three layers of the water column: (1) surface micro-layer (SML), (2) sub-surface layer and (3) bottom layer. Our measurements were distributed from the downstream end of Ho Chi Minh City to the South China Sea. We hypothesised that (i) the SML prokaryotic compartment would be enriched and more active than underlying waters, but rather similar or even reduced compared to the bottom water layer (enriched in suspended particles); and that (ii) the prokaryotic compartment and more particularly the EPA would vary at short spatial (a few kilometers) and temporal (within one month) scales in the estuary, due to short-term and spatial variations in suspended particulate matter (SPM) composition and

origin (David et al. 2019). Revealing short-term spatial and temporal variations in the ability of prokaryotes to mineralise OM and therefore making nutrients available for primary producers will contribute to understanding OM cycling in tropical estuaries.

3. Materials and methods

3.1. Study area

The Can Gio mangrove is located at the downstream end of Ho Chi Minh City (Southern Vietnam; ~13 million inhabitants) and flooded by the Saigon-Dong Nai River. The river discharges annually 37.4×10^6 m^3 of freshwater into the South China Sea and its basin covers a total catchment area of 40.6×10^3 km^2 (12% of the total terrestrial area of Vietnam; Ringler et al. 2002). The climate in Can Gio is monsoonal with a wet season from June to October and a dry season from November to May. Tidal amplitude is variable over time and ranges between 2 to 4 m depending on seasons and the distance from the sea (Nam et al. 2014). In 2000, the UNESCO designated the 719.6 km^2 of the Can Gio district as the first mangrove biosphere reserve in Vietnam and a land-use regulation was established. The north-west border of the mangrove is fringed by shrimp farms and salt producing lands, covering roughly 20% of the total reserve surface area, while the rest of the district is preserved from deforestation and mostly covered with mature trees of the species *Rhizophora apiculata*.

We selected the study sites on the main estuarine channel to be at the interface between land uses (Fig. 1): A) at the downstream end of Ho Chi Minh City (10°39'55"N 106°47'30"E); B) between shrimp farms and the forested mangrove area (10°34'19"N 106°50'11"E); C) in the centre of the mangrove

core area (10°31'04"N 106°53'13"E); D) and between the forested mangrove area and the South China Sea coast (10°29'32"N 106°56'55"E). At all these sites, the estuary has steep eroded banks, a depth of about 10-15 m and a width of about 600 m. Site E exhibited very different physical characteristics. It was located in a 1-3 m deep and 30 m large tidal creek within the mangrove core area, which does not receive freshwater upstream inputs (10°30'24"N 106°52'57"E; Fig. 1).

3.2. Sampling strategy

The study sites were sampled once per season, each one at one week intervals and in alphabetical order. Samplings were performed from January 14th to February 3rd 2015 during the dry season and from September 22nd to October 20th during the wet season. Exact sampling dates, corresponding tidal coefficient and tidal variations can be found in David et al. 2018. Salinity, pH and water temperature were measured at 5-min intervals during 24 h tidal cycles using a Yellow Spring Instrument® meter (YSI 6920) and dissolved oxygen (DO) was monitored similarly with a Hobo® data logger (HOBO U26-001), both immersed 30 cm below the water surface and calibrated before each survey.

We collected sub-surface water from the bow of a sampling boat using a 10 L plastic bucket immersed 10 cm below the water level. We sampled bottom water using a 2 L weighted bottle with a stopper pulled roughly 1 m above the bottom of the estuary. Samples from the surface micro-layer were taken using a glass plate sampler. The glass plate was immersed vertically and withdrawn at an approximate rate of 20 cm s^{-1}, following the recommendations of Harvey and Burzell (1972). It was then wiped between two polytetrafluoroethylene squeegees fixed face to face to remove the adhering SML, which was falling by gravity into a polyethylene bottle. This method was expected to recover the

first 60-100 µm of the water column, which constitutes a water-air interface where biological processes may differ from the rest of the water column. We repeated the procedure of dipping, withdrawing and wiping the plate to obtain a sufficient volume of sample for the analyses.

Sub-samples of water were vacuum-filtered through pre-combusted (5 h at 450°C) and pre-weighted glass fibre filters (Whatman® GF/F 0.7 µm). Suspended particulate matter concentration was measured gravimetrically after freeze-drying the filters. Three sub-samples for bacterial counting and cell size measurements were immediately preserved in 4.5 mL sterile Abdos Cryo Vials®, buffered with 0.2 µm pre-filtered formalin (4 % final concentration) and further stored at -25°C. Four and five replicates were analysed for EPA during the dry season and the wet season, respectively (Table 1). Replicates consisted of different water samples that were collected at 5-min intervals.

3.3. Sample processing

Preserved samples were defrosted at ambient temperature and filtered on 0.2 µm polycarbonate filters. Bacteria were stained using the 4'-6-diamino-2-phenylindole (DAPI) fluorescent stain, following the method described by Porter and Feig (1980). Ten to twelve randomly selected fields (to count at least 500 cells) were photographed using a Leica epifluorescence microscope coupled with a digital camera measuring light emission at 450 nm after excitation at 350 nm. Prokaryotes numeration and cell size measurements were performed with the open-source image-processing program ImageJ v1.5. We binarised monochromatic pictures to automatically count nearly circular aggregates of fluorescent pixels, which we assumed to be prokaryote cells. We measured cell size using a Feret box enclosing the cell and considering that the

cell diameter roughly equals the average distance between maximal and minimal Feret diameters (Loferer-Kröbbacher et al. 1998, Merkus et al. 2009).

The EPA was measured in the unfiltered fraction using the fluorigenic substrate analogue L-leucine-methylcoumarinylamide (Leu-MCA; Hoppe 1993). The substrate was added to 1.8 mL of water sub-samples and incubated on board during 1 h at *in situ* temperature and low natural light intensity. At the end of the incubation period, the enzymatic cleavage activity was stopped using Sodium Dodecyl Sulfate (1 % final concentration). Controls in duplicates were run similarly, except that the stopper solution was added before the substrate. The cleavage of Leu-MCA resulted from the exoproteolytic activity and was linearly related to the MCA fluorescence. Saturation curves were carried out to determine the saturating substrates concentration and 800 µM final concentrations were used for all samples. Since the substrate was saturating in our study, results corresponded to potential activity rates. The fluorescence was measured using a Varian Cary Eclipse spectrofluorometer (excitation/emission of 380/440 nm) and transformed to hydrolysis activity using a standard curve established with different concentrations of the fluorochrome MCA. All laboratory analysis were performed within 9 months after samples recollection.

3.4. Data analyses

We performed univariate multiple comparisons using non-parametric Kruskal-Wallis test due to variance heterogeneity and we identified groups of samples differing from one another using Wilcoxon pairwise comparisons (α = 5%, modified by Holm correction for multiple analyses). Statistical analyses and graphical representations were performed using R 3.3.2 (R Core Team 2017).

4. Results and discussion

Salinity along the 40 km of the Can Gio mangrove estuary ranged from 0 to 26; water temperature ranged from 26 to 31 °C; dissolved oxygen saturation ranged from 17 to 83 % and pH ranged from 6.5 to 7.8 (David et al. 2018, Taillardat et al. 2019). All parameters were linearly correlated to salinity, which increased from site A to site D and was roughly similar at sites C, D and E. Dissolved oxygen saturation and pH increased with salinity and water temperature decreased during the dry season and remained roughly stable during the wet season. Both pH and dissolved oxygen reflected the mixing of fresh and sea waters without significant perturbations induced by adjacent inputs (e.g. from mangrove creeks). The acidic pH values measured in the upper part of the estuary are likely due to the leaching of the surrounding acidic sulfate soils along with the decay of organic inputs (Thanh-Nho et al. 2018). The latter being also responsible for dissolved oxygen levels down to 1.2 mgO_2 L^{-1}. These organic inputs may originate from domestic and urban discharges of Ho Chi Minh City urban center and also from the industrial areas located along the Saigon and Dong Nai Rivers (Thanh-Nho et al. 2018)

4.1. Overall comparisons

Prokaryotic abundance ranged from 1.2 to 5.7 x 10^9 cells L^{-1} (Fig. 2). It was slightly above the values previously reported in other tropical (Bhaskar and Bhosle 2008) and sub-tropical mangroves (Bano et al. 1997, Williams and Jochem 2006). In tidal creeks of the Indus River delta, Bano et al. (1997) measured a prokaryotic abundance ranging from 1 to 4 x 10^9 cells L^{-1}. Bhaskar and Bhosle (2008) counted 0.6 to 3.5 x 10^9 cells L^{-1} in a mangrove dominated

estuary from the west coast of India. Williams and Jochem (2006) reported 0.27 to 2.91 x 10^9 cells L^{-1} in Florida Bay waterways.

The only recent record of exo-proteolytic activity in mangrove waters was provided by Williams and Jochem (2006) in Florida Bay, and to the best of our knowledge, no studies have ever been measuring EPA in Indo-Pacific mangrove waters. The activities we measured, ranging from 24 to 505 nmol L^{-1} h^{-1} in sub-surface waters (Fig. 2), were below those reported by Williams and Jochem (2006) in Florida Bay, which ranged from 70 to 1650 nmol L^{-1} h^{-1}. They were relatively similar to those measured by Rath et al. (1993) in an eutrophic mangrove-influenced station in a barrier reef off Belize (247 to 306 nmol L^{-1} h^{-1}). Converted to cell-specific activity, the EPA we measured in sub-surface waters ranged from 4.9 to 179.6 amol $cell^{-1}$, which is one order of magnitude below the values measured by Williams and Jochem (2006) in Florida Bay (52.2 to 1571.7 amol $cell^{-1}$) or by Rath et al. (1993) off Belize (316.0 to 765.7 amol $cell^{-1}$). Yet, the values of the present study were roughly similar to those reported by Lamy et al. (2009) after a *Phaeocystis globosa* bloom in the eastern English Channel (50 to 239.1 amol $cell^{-1}$). These results suggest that prokaryotes of the Can Gio mangrove estuary efficiently mineralise proteins and ease the availability of nitrogen for primary producers at a rate similar to that of other highly productive ecosystems.

4.2. Prokaryotic compartment stratification

No significant differences in prokaryotic abundance or cell size were measured between the three water layers during the dry season (Fig. 3), suggesting that the prokaryotic compartment is poorly stratified in the water column. Yet, lower EPA in the surface micro-layer compared to underlying waters and maximum values in the bottom (Fig. 3) suggest that prokaryotes

229 function differently between water layers. The Can Gio mangrove estuary is
230 highly hydrodynamic, with current velocity reaching 1.7 m s^{-1} during ebb and
231 1.3 m s^{-1} in the opposite direction during flood (David et al. 2018). A strong
232 vertical mixing is thus to be expected and probably inhibits the establishment
233 of a prokaryote-enriched SML, as previously observed in a highly hydrodynamic
234 estuary of Portugal (Santos et al. 2011). In a site of the Ria de Aveiro estuary
235 (Portugal), with similar hydrodynamic characteristics as ours, Santos et al.
236 (2011) measured a similar abundance of prokaryotes between water layers but
237 lower prokaryotic productivity in the SML compared to underlying waters,
238 while the opposite was recorded when a structured SML could be established
239 due to lower hydrodynamism. We suggest that although prokaryotes may be
240 homogenously distributed in the upper layers of the water column in highly
241 hydrodynamic estuaries, they are less active at the water-air interface. Our
242 hypothesis that the SML would be more active than underlying waters is thus
243 invalidated, most probably because of the high hydronynamism of the Can Gio
244 mangrove estuary.

245 Higher EPA and suspended particulate matter concentrations in the
246 bottom compared to other layers (Fig. 3) suggest a particle-associated
247 behaviour of prokaryotes in bottom waters. Actually, prokaryotic cells are
248 influenced by the presence of particles in the water column, creating patches,
249 or “hotspots” of biomass where OM is intensely recycled (Long and Azam 2001,
250 Simon et al. 2002). In a tidal estuary exhibiting high loads of suspended matter,
251 particle-attached prokaryotes can reach up to 80% of total abundance (De
252 Souza et al. 2003). It has previously been observed that particle-attached
253 prokaryotes were more active than free-living cells (Grossart et al. 2007a,
254 2007b, Schapira et al. 2012). Although in our study no difference could be
255 evidenced in prokaryote abundance between water layers, the proportion of

256 particle-attached cells may have been higher in the bottom compared to other
257 layers, due to higher loads of SPM (Fig. 3). Actually, a previous study (David et
258 al. 2019) showed a high relative contribution of branched fatty acids, indicative
259 of bacteria, in short settling-time particles, which are more hardly resuspended
260 and tend to remain closer to the estuary bottom. We thus suggest that short
261 settling-time particles are the sites of an intense prokaryotic
262 activity. Prokaryotes may however alternate from a free-living and a particle-
263 attached lifestyle (Grossart et al., 2010, Riemann and Winding, 2001) and both
264 populations may belong to the same community as no difference in cell size
265 was revealed between water layers (Fig. 3). Our hypothesis that the bottom
266 layer would be more active than upper waters is therefore confirmed, most
267 probably because of higher loads of SPM compared to other water layers.
268
269 4.3. Link with organic matter quality

270 During the dry season, EPA was roughly 10 times higher than during the
271 wet season, while prokaryotic abundance did not differ and cells were only
272 slightly smaller (Fig. 2), which clearly indicates that the prokaryotic biomass did
273 not notably vary between seasons. We thus expect the specific activity of
274 prokaryotes to vary spatially and/or temporally rather than their abundances.
275 Exo-proteolytic activity is usually closely related to the prokaryotic abundance
276 (Cunha et al. 2000, Patel et al. 2000) and thus, seasonal stability in bacterial
277 stocks is surprising. Yet, intense top-down pressure (grazing or virus mortality)
278 could explain a decoupling between prokaryotic standing stock and activity
279 (Fuhrman and Noble 1995, De Souza et al. 2003, Pradeep Ram et al. 2018).
280 Aspects of top-down processes on prokaryotes would require further
281 investigations in mangrove ecosystems.

In the Can Gio mangrove estuary, organic matter exhibited higher lability during the wet season, associated with a higher proportion of polyunsaturated fatty acids indicative of fresh phytoplankton-derived OM, while during the dry season, OM sources were mostly terrestrial (David et al. 2019). The observed seasonal EPA variability could thus be related to the seasonal variation in the composition of organic pools. Moreover, previous studies demonstrated significant protease stimulation in the presence of terrestrial dissolved OM (Traving et al. 2017) and humic-rich dissolved OM (Stepanauskas et al. 1999). Baltar et al. (2017) demonstrated experimentally that prokaryotes fuelled with mangrove-derived dissolved OM increased their EPA up to 400 nmol L^{-1} h^{-1} after 4 days of incubation. In our study, the renewal of water in the mangrove was most probably higher during the wet season compared to the dry season, due to rain inputs and a higher discharge of the estuary. Thus, OM released by mangrove leaves could be more rapidly diluted than during the dry season. Higher leaching of mangrove leaves was actually measured in the Can Gio mangrove during the wet season (Vinh et al. 2020). As a consequence, the EPA did not increase to the same levels as recorded during the dry season, characterized by heavier loads of OM of terrestrial origin. Similarly, high EPA at site E during the wet season in comparison to the other sites (Fig. 2) was most likely due to the high concentrations of mangrove-derived OM nearby the mangrove forest. The ability of prokaryotes to mineralise OM thus varies at the seasonal scale most probably as a response to OM quality (*i.e.* origin, lability).

Finally, the high EPA at site B in sub-surface water during both seasons at high tide (Fig. 2) compared to its relative stability in other sites (except site E) whatever the tidal stage considered suggest that OM differed at this site. Site B is located down the area dedicated to shrimp farming (Fig. 1) and the intensive shrimp production requires punctual water renewal in ponds, releasing

wastewaters loaded with OM and nutrients to adjacent ecosystems (Anh et al. 2010). Shrimp pond effluents are constituted by fresh and highly labile OM that stimulates bacterial growth (Vivier et al. 2019), which may explain the higher EPA activity measured at site B. Our results suggest that both mangrove-derived OM and shrimp pond effluents increased the release of extracellular enzymes by prokaryotes. The aim of such enzymes is to convert compounds of high molecular weight into monomeric substances, allowing them to be transported through the cytoplasmic membrane. It is thus to be expected that a higher degree of macromolecules polymerisation or organically less labile OM would lead to a higher release of extracellular enzymes by prokaryotes. Baltar et al. (2017) rather suggested that it is the high palatability (that is probably linked to easier assimilation) of mangrove-derived dissolved OM that enhances extracellular enzymes production. According to Findlay et al. (1991), autochthonously produced OM, including exudates, intracellular contents and biomass of primary producers are easily degraded by prokaryotes, while allochthonous sources are relatively more complex and refractory to prokaryote degradation. Our results suggest that EPA is enhanced in both situations: when OM is mostly composed of macromolecules resistant to degradation, and when OM is constituted by unusually high amounts of fresh and labile OM.

4.4. Prokaryotes behaviour in mangrove ecosystems

Conceptually, there might be a “common” situation for prokaryotes in mangrove ecosystems, where inputs of fresh labile organic matter are sufficient to sustain their nitrogen demand. In the Can Gio mangrove this “common” situation occurs during the wet season. It is probably reinforced by the fact that the strong anthropogenic pressure brings easily available nitrogen

to the ecosystem (Nguyen et al. 2019). Consequently, prokaryotes release low amounts of exo-proteolytic enzymes. The situation becomes "uncommon" during the dry season, when fresh and labile OM is getting more scarce. Nitrogen demand is then fullfiled through higher release of exo-proteolytic enzymes. Another "uncommon" situation is the input of OM from shrimps farms, that may modify nutrient balance, and stimulate prokaryotes release of exo-proteolytic enzymes. Yet, the focus on one mangrove ecosystem is insufficient to conclude with certainty on prokaryotes behavior in these understudied type of ecosystems (regarding prokaryotes) that are mangroves. Further studies in other regions would need to confirm these preliminary results in other regions, especially regarding anthropogenic releases that may disturb the system and switch it to an unusual fonctionning. Later on, if this switch is broadly confirmed in other ecosystems, its consequences on trophic webs and nutrient cycling should be assessed.

5. Conclusions

To the best of our knowledge, this study is the first to measure exo-proteolytic activity in Indo-Pacific mangrove waters. The surface micro-layer of the estuary, that we expected to serve as a receptacle for anthropogenic pollutants and organic matter, is not well structured because of high water turbulence. Yet, higher loads of suspended particulate matter in bottom waters were correlated to higher EPA in this layer. The nature of OM transported by the Can Gio mangrove estuary may affect the ability of prokaryotes to degrade OM and recycle nitrogen, despite abundance and size of prokaryotic cells did not vary. Releases of extracellular enzymes seem to be increased as a response to "uncommon" situations induced by the input of fresh and labile OM (*e.g.*

362 shrimp farm effluents) or by the lack of labile OM and the need to hydrolyse
363 refractory compounds (*e.g.* during the dry season).
364

365 **6. Acknowledgments**

366 The authors would like to thank Xavier Mari from the French IRD for
367 providing the glass plate sampler during field trips and all Vietnamese students
368 for their help during samplings. The support of the “Projet Exploratoire Premier
369 Soutien” (PEPS CNRS-IRD “Mangrove”) grant is also greatly acknowledged.

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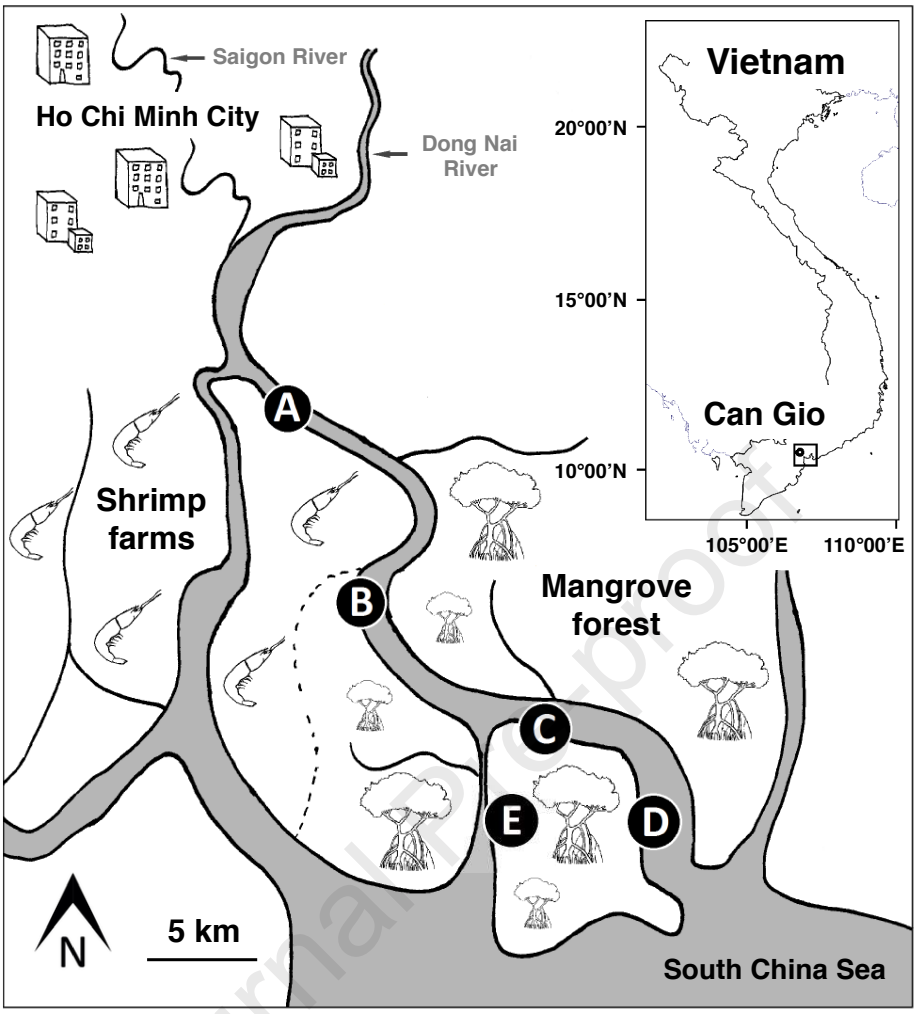

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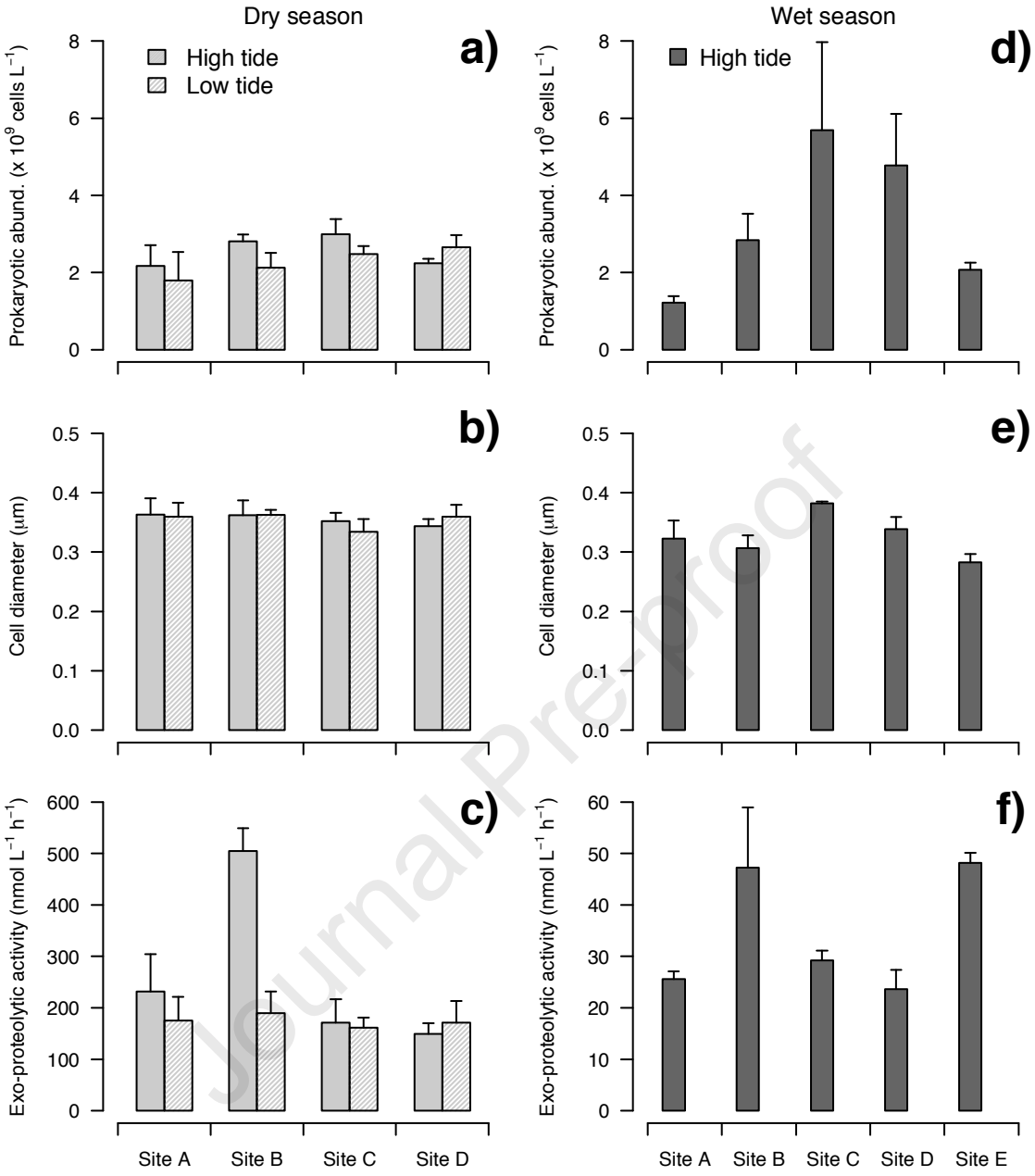
510 Fig. 1: Map of the sampling area in the Can Gio mangrove (Southern Vietnam).

511 A, B, C, D and E indicate the sampling stations along the estuary and within the

512 tidal creek



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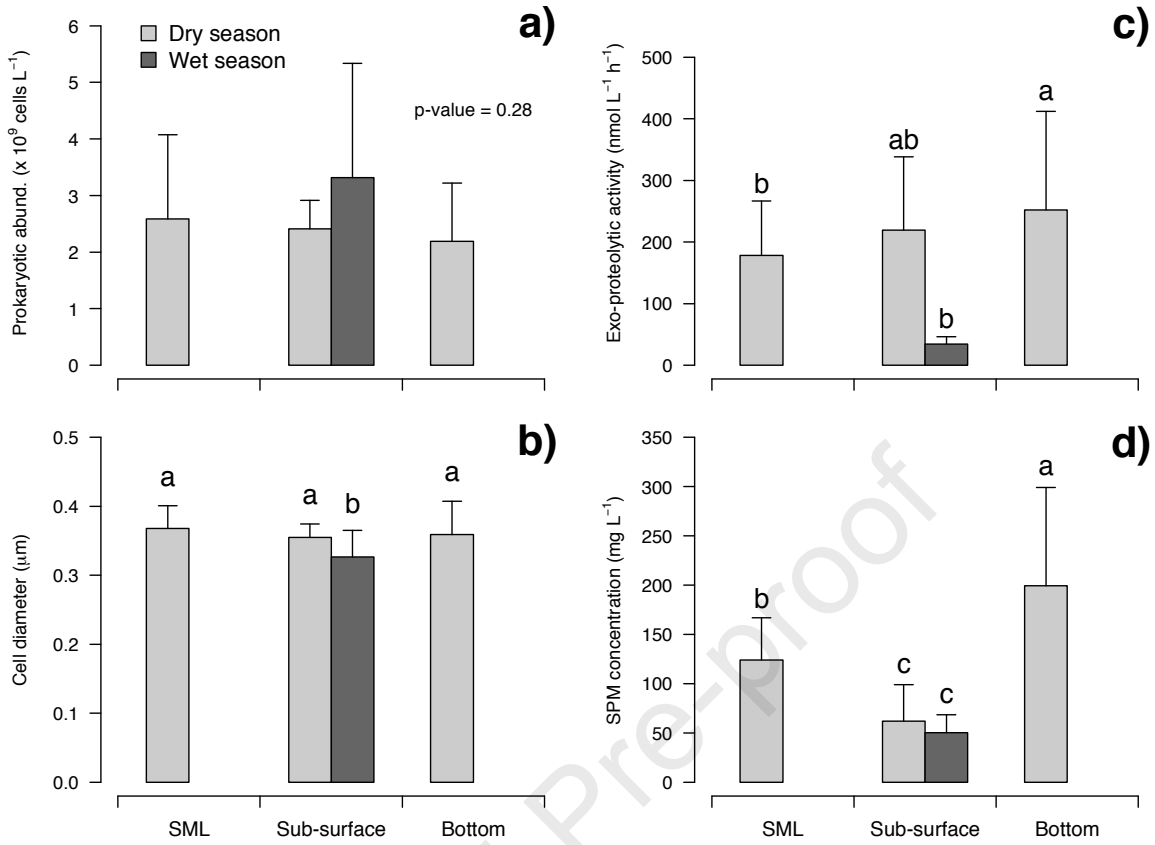
514 Fig. 2: Spatial variability in a) & d) prokaryotic abundance b) & e) cell diameter
515 and c) & f) leucine-aminopeptidase exo-proteolytic activity in the sub-surface
516 water layer of the Can Gio mangrove, during dry season (left panel) and wet
517 season (right panel). For a), b), d) and e) n = 3 for each bar; for c), n = 4 for each
518 bar; and for f), n = 5 for each bar. Statistical differences between groups were
519 not tested given the low amount of data.



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521 Fig. 3: Vertical variability in a) prokaryotic abundance b) cell diameter c)
522 leucine-aminopeptidase exo-proteolytic activity and d) suspended particulate
523 matter concentration in the three water layers of the Can Gio mangrove. For a)
524 and b), n = 24 for each bar during the dry season and n = 15 during the wet
525 season, and for c) and d), n = 32 for each bar during the dry season and n = 25
526 during the wet season. Letters indicate significant differences at α = 5%
527 (Kruskal-Wallis rank test plus Wilcoxon pairwise comparisons with Holm-
528 corrected α).

529 Table 1: Sampling plan and sample distribution

Season	Study sites	Water layers	Tidal stage	EPA and SPM			Cell counts and size		
				Replicates[a]	Total per layer	TOTAL[b]	Replicates[a]	Total per layer	TOTAL[b]
Dry	A, B, C, D	SML, sub-surface, bottom	High tide + low tide	4	32	96	3	24	72
Wet	A, B, C, D, E	Sub-surface	High tide	5	25	25	3	15	15

[a]Replicates are different water samples collected at 5-min intervals

[b]TOTAL is the total number of samples

530

Highlights

- Exo-proteolytic activity is similar to that of other highly productive ecosystems
- Bottom waters are more active that upper layers, especially the surface micro-layer
- Exo-proteolytic activity is enhanced by the input of labile organic matter
- The need to hydrolyse refractory compounds increases exo-proteolytic activity