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Mangrove microbial diversity and the impact of trophic contamination

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A B S T R A C T

Mangroves are threatened ecosystems that provide numerous ecosystem services, especially through their wide biodiversity, and their bioremediation capacity is a challenging question in tropical areas. In a mangrove in Mayotte, we studied the potential role of microbial biofilm communities in removing nutrient loads from pre-treated wastewater. Microbial community samples were collected from tree roots, sediments, water, and from a colonization device, and their structure and dynamics were compared in two areas: one exposed to sewage and the other not. The samples from the colonization devices accurately reflected the natural communities in terms of diversity. Communities in the zone exposed to sewage were characterized by more green algae and diatoms, higher bacteria densities, as well as different compositions. In the area exposed to sewage, the higher cell densities associated with specific diversity patterns highlighted adapted communities that may play a significant role in the fate of nutrients.

1. Introduction

Recent assessments suggest that about one-third of mangrove, sea grass and salt marsh areas around the world have already been lost over recent decades as a result of reclamation, deforestation, engineering and urbanization (Lewis et al., 2011; Peixoto et al., 2011; Penha-Lopes et al., 2011), as well as transformation to provide aquaculture ponds (Alongi, 2002). Many coastal lagoons in the tropics support dense mangrove forests, which are productive areas harboring a wide diversity of organisms. Adapted to intertidal zones, they are subjected to highly variable physicochemical conditions of salinity, flooding, light, and temperature, which give rise to the high diversity that characterizes mangrove ecosystems (Feller et al., 2010).

Mangroves are often established on nutrient-rich sediments, and are able to absorb excess nutrients without suffering any major structural or functional disturbance (Saenger, 2002). Nedwell (1975) was the first to show that pretreated wastewater discharge into a mangrove swamp in Fiji reduced eutrophication in adjacent coastal waters, and therefore suggested that mangroves might serve as the final stage in sewage treatment. Since then, most attempts to evaluate the potential role of mangrove to remove nutrients from sewage have been done in the form of experimental studies (Chu et al., 1998; Wu et al., 2008), or on constructed pilot sites (Yang et al., 2008; Tam et al., 2009). Yang et al. (2008) highlighted the need for complementary treatment to eliminate pathogenic bacteria. However, only a few studies have investigated natural mangrove sites in intertidal zones (Wang et al., 2010; Penha-Lopes et al., 2011). Wang et al. (2010) explored changes in water quality in a subtropical mangrove estuary (China), and highlighted the fact that large quantities of nutrients may be trapped by the mangrove during flood periods. In a comparison of non-impacted and peri-urban subtropical mangroves (Mozambique), Penha-Lopes et al. (2011) showed that both the structure and the fitness of a natural shrimp population (*Palaemon concinnus*) were impacted by nutrient levels. Tam showed as early as (1998) that adding wastewater to mangrove soils seems to stimulate the growth of microbial populations, probably because of the nutrients and carbon supplied in wastewater, but this compartment has not been thoroughly explored within the framework of domestic-sewage discharge in mangroves. Estuarine waters are dynamic environments in which sediments, and marine and fresh water mix, resulting in salinity and nutrient gradients. Shifts in physical, chemical, and microbiological properties between freshwater and adjacent coastal marine environments occur over short periods of time, driven by tides and freshwater flows, which create an intense abiotic pressure that influences the composition of bacterioplankton communities (Crump et al., 1999). Microorganisms have large

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population sizes and display long dispersal distances, high reproductive rates, and remarkable genetic diversity, suggesting that they can cross environmental boundaries, including salinity, more readily than multicellular organisms (Logares et al., 2009).

On solid surfaces, microorganisms organize themselves into microbial biofilms, mainly consisting of heterotrophic bacteria and autotrophic eukaryotes that are often known collectively as the "microphytobenthos", which is embedded in a microbial organic matrix (Decho, 2000). These biofilm communities are present in the upper layers of sediment (Holguin et al., 2001) as well as on tree roots (Toledo et al., 1995; Gomes et al., 2010), where they carry out a number of different functions, including nutrient transformation, sediment stabilization, plant-growth promotion, and even providing protected suitable areas for pathogens entering marine systems. The impacts of pollutants such as polycyclic aromatic hydrocarbons (PAH, for a review see Fernandez-Luqueno et al. (2011)) and phthalates have been studied in mangrove ecosystems. Conversely, changes in trophic conditions due to nutrient loading have received much less investigation in these tropical zones (Underwood, 2002; Ramanathan et al., 2008), unless they are threatened by urban sewage or aquaculture. Yet such changes, which have been extensively studied in freshwater ecosystems, are known to modify the functionality of biofilm communities, as well as their diversity (e.g. Pesce et al., 2008; Berthon et al., 2011).

This study focuses on biofilm microbial communities in a tropical mangrove with two different trophic statuses, either exposed or not exposed to effluents from a domestic wastewater pre-treatment device. We tested an experimental approach using artificial substrates to collect natural biofilms, in order to avoid sampling biases due to environmental heterogeneity. Microbial communities were also sampled *in situ* to collect pelagic, benthic and root biofilms in order to compare their structures and diversities. This approach provides a very useful way of extending our knowledge about the microbial communities associated to mangrove ecosystems, and of evaluating the impact of urban sewage on these communities.

2. Materials and methods

2.1. Study sites

Two sites in a mangrove located in Chirongui bay, southwest of Mayotte Island in the Indian Ocean (12°55′S, 45°09′E, Fig. 1) were investigated in this study. This area was described in a previous study by Herteman (2010). Domestic wastewater collected from the contiguous village of Malamani (400-equivalent inhabitants) was subjected to primary treatment in a sedimentation tank which reduced the suspended matter concentration by 50%. Pre-treated wastewater was then discharged at low tide into a 45 m × 15 m mangrove zone dominated by *Rhizophora mucronata,* at the rate of 10 m^3 per 24 h. This site, designated the "impacted plot", was studied simultaneously with a "reference plot" located in the same mangrove zone, but not subjected to wastewater disposal. Our study was conducted in March 2009 at the end of the rainy season, during the spring tides phase, after this wastewater pre-treatment and discharge had been taking place for 1 year.

2.2. Sub-surface and porewater sampling

Salinity and pH were measured directly on the field in crab water holes (0 cm) and in deep piezometers (30 and 100 cm). Mean values were obtained from two-days measurements under similar hydrological conditions. Nutrient contents were measured 0.45-μm filtered water sampled at the same depth, and transported to the analytical laboratory (ARVAM, Reunion, France) in a cool-box at 4 °C. Nutrients concentrations were determined following French standard operating procedures (AFNOR). Interference with high NaCl concentrations was avoided using blank samples on a salinity gradient. In addition, the carbon source level was investigated through the chemical oxygen demand (COD) and biological oxygen demand (BOD) of the pre-treated wastewater that were measured three times during the first year of wastewater pre-treatment.

2.3. Collection of microbial communities

Six large frosted glass slides (47 cm^2 per slide) fixed in a perforated plastic box were used as artificial substrata allowing periphytic communities to colonize them (Morin et al., 2007). One box was placed in each plot (the impacted plot and the reference plot) at soil level, and maintained with ropes to arching stilt roots of *R. mucronata* for 8 days before sampling. This corresponded to a high tide period during which the sampling system remained under water for about 40% of the time. At the end of this period, the 6 slides from both of the sampling sites were collected, and transported to the field laboratory within 2 h in a cool-box. Colonized biofilms were then carefully removed from each replicate glass slide separately, using a razor blade, and suspended in

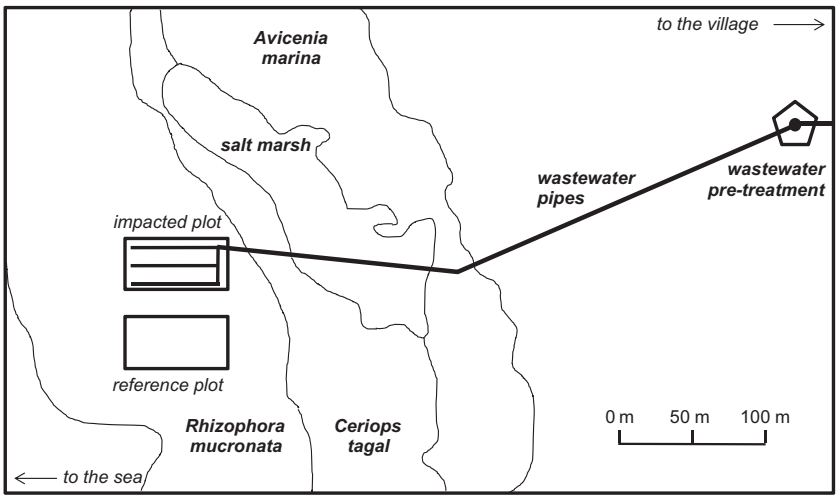

Fig. 1. Mangrove study area and land cover on Mayotte Island in the Indian Ocean.

100 mL of 0.2 μm-filtered mangrove water. Functional measurements were performed immediately, and structural and diversity measurements were carried out subsequently on frozen material.

Microbial communities were also sampled from sediments, water, and natural biofilms from both plots at two dates (the beginning and end of the 8-day biofilm colonization period, respectively). Superficial sediments were sampled on the first two centimeters using a truncated syringe. Two replicates of 2 mL of sediments were randomly collected at each date from each plot (8 samples). Surface water was sampled with a sterile bottle from small natural pools at each date and from each plot (4 samples, 20 mL each). Root biofilms were sampled from the bark of aerial *R. mucronata* stilt roots just above soil level. Three samples of approximately 8 cm^2 of root bark were collected at each date from each plot (12 samples), and transported to the field laboratory in a cool-box. Natural root biofilms were then carefully removed from each bark sample and suspended in 10 mL of 0.2-μm filtered mangrove water. All samples were prepared (by being frozen or kept in liquid nitrogen) for later structural and diversity measurements.

2.4. Characterization of microbial communities

2.4.1. Fluorescence measurement

The phototrophic community in colonized biofilms was characterized using various different photosynthesis parameters measured using a Phyto-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany) equipped with the Optical Unit ED101-US (measuring chamber) featuring a standard quartz cuvette. Before measurements, triplicate colonized biofilm suspensions were dark-adapted for 30 min. A 2-mL sample was then introduced into the measuring chamber and allowed to stabilize under modulated (non-actinic) light for a couple of minutes. The basal fluorescence of dark-adapted samples (Fo) was measured as a valid proxy for the Chl *a* biomass estimation. A short saturating pulse was then applied (wavelength peak: 655 nm; intensity: 4000 μmol photons m^{-2} s^{-1}; duration: 200 ms) to induce the maximal fluorescence yield (Fm) from dark-adapted cells. The variable fluorescence (Fv = Fm − Fo), and the maximum efficiency of photosystem II, (ΦPSII = Fv/Fm), were then calculated, the second one being is used as a proxy of the sample's fitness (Parkhill et al., 2001). The fluorescence signals obtained at four different excitation wavelengths (470, 520, 645 and 665 nm, channels F1, F2, F3 and F4, respectively) depend on pigment composition. The F1 signal is mainly linked to diatoms and green algae, whereas the F2 is related to diatoms only. The F3 signal is linked to the Cyanobacteria, while the F4 is related to the whole algal community (Jakob et al., 2005). In order to assess the pigment dominance of the phototrophic community of colonized biofilm samples indirectly, we used the ratios of the fluorescence of dark-adapted samples associated with channels F1, F2 and F3 to the fluorescence associated with channel F4. Following Izagirre et al. (2009) the F1/F3 ratio was also used as an indication of dominance of green algae + diatoms *versus* Cyanobacteria.

2.4.2. Abundance of bacterial cells

Colonized biofilm suspensions (1.8 mL) from three replicates in each plot were preserved using 100 μL of 0.22-μm filtered 37% formalin in 2-mL cryovials, and immediately frozen inliquid nitrogen (−196 °C). Bacterial counts were performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an air-cooled argon laser (488 nm, 15 mW). Stained bacterial cells, excited at 488 nm, were counted from their right-angle light scatter (RALS) and green fluorescence (FL1) collected at 530/30 nm. These cell parameters were recorded on a four-decade logarithmic scale mapped onto 1024 channels. Fluorescent beads (0.94 μm, Polysciences Inc., Warrington, PA, USA) were added to each sample as an internal standard. Standardized RALS and FL1 values (sample RALS and FL1 divided by 0.94 mm-beads RALS and FL1, respectively) were used as an estimation of the relative size and nucleic acid content of bacterial cells, respectively (Troussellier et al., 1999). Bacterial cells tend to cluster into two distinct fractions based on differences in individual cell fluorescence (related to their nucleic acid content) and in the side and forward light scatter signal (related to their cell size). These fractions are defined as HNA cells (high nucleic acid content) and LNA cells (low nucleic acid content), respectively (see details in Bouvier et al., 2007).

2.4.3. Abundance of phytobenthic cells

Eukaryotic cells below the 20-μM size class (nano- and picoeukaryotes) in colonized biofilms were also counted with the same flow cytometer. Cells excited at 488 nm were detected and counted on the basis of their RALS properties, and their orange (585/42 nm) and red fluorescence (>650 nm) emissions, which are related to phycoerythrin and chlorophyll pigments, respectively. Fluorescent beads (0.94 μm) were also added to each sample as an internal standard. Cell concentrations were calculated as for the bacterial community.

2.4.4. Community level molecular fingerprinting

Water samples and colonized and natural biofilm suspensions were filtered through a 0.2-μm Nuclepore filter (Whatman). Nucleic acid extraction for sediment and biofilm samples was performed using the "Ultraclean for Soil" kit and "PowerBiofilm" DNA Isolation Kit (MoBio) respectively, according to the Manufacturer's instructions. For the water samples, after lysing the cells, nucleic acids were co-precipitated using GenElute™-LPA (Sigma–Aldrich) according to the Manufacturer's instructions, and dissolved in TE buffer. The integrity of the total DNA was checked using a NanoDrop 1000 spectrophotometer (Thermofisher Scientific). Polymerase Chain Reactions (PCRs) of the eukaryotic 18S rRNA and of the bacterial 16S rRNA gene fragments were performed using 15 ng of template DNA. Primers Euk1Af (Sogin and Gunderson, 1987) and Euk516r-GC (Amann et al., 1990) were used to amplify a 560-bp fragment of the 18S rRNA gene, while primers 358f-GC (Muyzer et al., 1993) and 907rM (Schauer et al., 2003) were used to amplify a 570-bp fragment of the 16S rRNA gene. All amplifications, including a negative control, were performed as described in Dorigo et al. (2007).

PCR samples were analyzed by DGGE with an INGENYphorU-2∗2 system (Ingeny International), using a 1-mm thick gel of 6% (w/v) polyacrylamide with a denaturing gradient, where 100% denaturant contained 7 M urea and 40% formamide. The eukaryotic and prokaryotic communities were studied with 35–65% and 40–80% gradients respectively. Nucleic acids were detected by UV transillumination on stained DGGE gels (SYBR Gold, Molecular Probes). As we were focusing on the prokaryotic community, all 36 samples (colonized and natural biofilms, sediment, and water samples) were studied on the same DGGE gel, whereas the eukaryotic and prokaryotic communities were studied separately in the 12 samples from the colonized biofilms. DGGE profiles were analyzed using GelCompar II software (Applied Math NV) leading to a simple matrix of the presence/absence of bands (prokaryotic community for all samples). For colonized biofilms, a more detailed analysis was carried out, leading to a matrix of the quantification of band intensities (eukaryotic and prokaryotic communities of colonized biofilms).

2.5. Statistical data analysis

The richness (number of species per sample) and Shannon index were estimated from the DGGE bands.

The prokaryotic diversity of all samples was compared on the basis of the DGGE presence/absence matrix using the Jaccard

similarity index. The similarity matrix was used to perform Multi-Dimensional Scaling (MDS) analysis.

The diversity of colonized biofilm communities (prokaryotic and eukaryotic) was compared on the basis of the DGGE matrix of relative band intensities submitted to Principal Component Analysis (PCA).

The abundances, fluorescence measurements, and diversity index of the colonized biofilms were tested for a plot effect using a one-way ANOVA with a 5% threshold. Parameters showing a significant plot effect were then tested using a Student's t test.

All these statistical tests were carried out using XLSTAT Software (version 2011.1).

3. Results

The impacted and reference plots shared common characteristics for temperature (27.4 ± 0.04), pH (6.62 ± 0.03), salinity (34.5 ± 1.5) and cohesive sediment structure, (mostly silt: 76%; fine sand: 21%, clay: 3% at surface). However, they differed with regard to nutrient concentrations (Table 1). In the surface layer of the sediment (0–30 cm), NO_3^-, NO_2^- and NH_4^+ concentrations in the interstitial water were higher in the impacted plot than in reference plot, whereas no difference was observed in the deeper layers. Conversely, the PO_4 concentration was higher in the deeper layers of the impacted plot, whereas no difference was observed in the surface layers. Concerning the carbon substrate, a mean COD value of 167 ± 57 mg L^{-1} was observed for the pre-treated effluent with a relatively high COD/BOD ratio (2.2 ± 0.4), indicating that organic matter was loaded in the impacted plot by the effluent, but this organic matter was poorly biodegradable.

3.1. Microbial community structure

In the impacted plot exposed to wastewater disposal, the total bacterial cell density was significantly higher than the one reported in the reference plot ($p = 0.018$), in particular for the fraction with high nucleic acid content (HNA bacteria, $p = 0.016$), which are considered to consist of more active bacteria (Table 2). Conversely, the proxy associated to the biomass of the phototrophic community (basal fluorescence measured on dark-adapted samples by the Phyto PAM) did not reveal any significant difference between the two zones ($F = 0.0967$; $p = 0.762$). Different fluorescence ratios were used to carry out an indirect assessment of pigment dominance in the phototrophic community. Among these ratios, F1/F3 and F1/F4 revealed significant differences between the communities originating from the two plots ($p = 0.026$ and $p < 0.001$, respectively), highlighting the differing contributions made by green algae and diatoms to the biomass of the periphytic communities (Fig. 2). The contribution of green algae and diatoms was higher in the impacted plot than in the reference one. We did not observe any significant difference between the photosynthetic performances of biofilms from our slides from one plot to another ($F = 0.00207$; $p = 0.965$). However, the maximum efficiency of photosystem II values (ΦPSII below 0.3) were unusually low in samples from both plots.

Table 1
NO_3^-, NO_2^-, NH_4^+ and PO_4 concentrations in nM in sediment interstitial water.

	Impacted plot			Reference plot		
	0 cm	30 cm	100 cm	0 cm	30 cm	100 cm
NH_4^+	5.04	14.30	2.64	2.07	0.52	2.48
NO_3^-	4.13	5.95	3.53	4.16	2.45	4.53
NO_2^-	0.37	0.26	0.12	0.47	0.08	0.12
PO_4	3.7	2.7	8.8	2.4	0.7	2.9

3.2. Community level molecular fingerprinting

For prokaryotic community fingerprinting, all 36 samples (colonized and natural biofilms, sediment, and water) were studied on the same DGGE gel. The diversity indices based on these data are summarized in Table 3. A total of 51 different DGGE-Operational Taxonomic Units (DGGE-OTUs) were detected in the 36 samples. On average, 26, 16 and 10 DGGE-OTUs were identified in the sediments, biofilms, and water, respectively. Sediment and colonized biofilms displayed the highest species richness and diversity (Shannon Index). The MDS values based on these DGGE data are presented in Fig. 3. The MDS values found for sediment and water communities were sharply distinguished from each other, while those found for root biofilm communities were more scattered. Impacted and reference natural samples remained closely clustered. Colonized biofilm communities appeared at an intermediate position between the natural samples. They exhibited a clear discrimination between impacted and reference samples.

With regard to the prokaryotic community of colonized biofilms (12 samples), a total of 33 different DGGE-OTUs were detected, with an average of 19 DGGE-OTUs per sample for both the reference and impacted plots. The diversity indices based on these data are summarized in Table 3. No significant difference was detected for the diversity index (ANOVA, data not shown) between the impacted and reference communities. Fig. 4a reports the PCA analysis of prokaryotic diversity showing the ordination of sampling points with respect to the two principal axes. The first two axes (PC1 and PC2) accounted for more than 61% of the variance. PC1 appeared to be related to the origin of the plots, with a clear separation between samples from the impacted plot and those from the reference plot. The reference samples appeared to be more widely scattered along PC2 than the impacted ones.

For the eukaryotic community of colonized biofilms (12 samples), a total of 60 DGGE-OTUs were detected, with an average of 23 and 25 DGGE-OTUs per sample for the reference and impacted plots, respectively. Diversity indices based on these data are summarized in Table 3. No significant difference in the diversity index (ANOVA, data not shown) was detected between the impacted and reference communities. Fig. 4b reports the PCA analysis of eukaryotic diversity showing the ordination of sampling points with respect to the two principal axes. The first two axes (PC1 and PC2) accounted for more than 32% of the variance. PC1 also appeared to be related to the origin of the samples per plot, as shown in Fig. 4a.

4. Discussion

This study reports for the first time the microbial diversity of a mangrove in the tropical island of Mayotte which suffers anthropogenic impacts due to a demographic explosion. The differences found in the community composition add to our knowledge about microbial distribution in mangrove ecosystems. The loading of nutrients in aquatic ecosystems has often been reported to be a cause of functional, structural and diversity changes in biofilm microbenthic communities in freshwater ecosystems (e.g. Underwood et al., 1998; Pesce et al., 2008), but relatively rarely in intertidal marine environments (Chu et al., 1998; Underwood, 2002; Ramanathan et al., 2008). We first had to validate the use of artificial substrates to collect recent biofilms as an efficient tool for the sampling and observation of biofilm communities in the mangrove. We then used the results obtained to characterize the biofilm community and demonstrate its possible links with nutrient concentrations.

4.1. Sampling microbial communities in the mangrove

The samples of microbial communities (bacteria and microalgae) were taken both from natural substrata and from an artificial

Table 2
Cell abundances in cells mL^{-1} measured on colonized biofilms and ANOVA *p*-value. Data are cell number mean values ± standard deviation. Significant differences, at 5% threshold, between impacted and reference plots are indicated in bold type.

	Cell abundances (cells mL^{-1})		ANOVA *p*-value
	Impacted plot	Reference plot	
HNA bacteria	**$1.0 \times 10^6 \pm 1.8 \times 10^5$**	**$5.1 \times 10^5 \pm 1.3 \times 10^5$**	**0.016**
LNA bacteria	$3.3 \times 10^5 \pm 1.0 \times 10^4$	$4.0 \times 10^5 \pm 1.8 \times 10^4$	0.333
Total bacteria	**$1.3 \times 10^6 \pm 1.3 \times 10^5$**	**$9.1 \times 10^5 \pm 1.5 \times 10^5$**	**0.018**
Cyanobacteria	$7.8 \times 10^1 \pm 3.8 \times 10^1$	$8.0 \times 10^1 \pm 2.1 \times 10^1$	0.925
Picoeukaryotes	$2.5 \times 10^3 \pm 1.1 \times 10^3$	$1.5 \times 10^3 \pm 2.5 \times 10^2$	0.231
Nanoeukaryotes	$8.9 \times 10^2 \pm 4.4 \times 10^2$	$6.5 \times 10^2 \pm 9.1 \times 10^1$	0.411

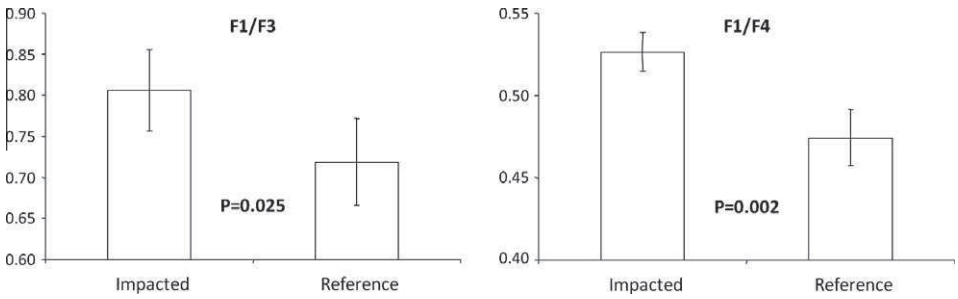

Fig. 2. Fluorescence ratios of dark-adapted biofilm samples from colonized biofilms from the impacted and reference plots.

Table 3
Species richness and Shannon diversity index deduced from DGGE fingerprinting of prokaryotic and eukaryotic communities of mangrove samples from sediment ($n = 8$), water ($n = 4$), root biofilm ($n = 12$), and colonized biofilm ($n = 12$). Data are mean values ± standard deviation.

	Richness	Shannon index
Prokaryotic community		
Sediment	25.8 ± 2.3	3.086 ± 0.116
Water	10.3 ± 1.5	2.159 ± 0.115
Root biofilm	12.8 ± 3.3	2.279 ± 0.285
Colonized biofilm	19.3 ± 1.4	2.677 ± 0.144
Eukaryotic community		
Colonized biofilm	24.2 ± 2.7	2.692 ± 0.203

glass substrate. Natural communities were also collected from several different compartments: water, sediments and roots.

Due to the tidal rhythm in the mangrove, it was necessary to avoid as much as possible differences related to temperature and desiccation stresses, which are known to be prime factors controlling intertidal biofilms (Kaehler and Williams, 1997). For this reason, the colonization time could not exceed 8 days, which corresponds to a spring tide period. By their functional ability, evaluated from a photosynthesis process with low ΦPSII values (below 0.3), the photoautotrophic communities presented the functional indexes of a recent stage. Values higher than 0.5 indicate a satisfactory physiological acclimation to the growth conditions (Parkhill et al., 2001; Claquin et al., 2010), and non-limited algae presented ΦPSII values of around 0.6–0.7 (Kromkamp and Peene, 2005). After one week of colonization time, the phototrophic community did not yet seem to be functioning efficiently, unlike the mature communities harvested from root barks, which had higher ΦPSII values (0.4 ± 0.1). Functional evaluation of photosynthesis proved to be too closely linked to the stage of biofilm development to be used

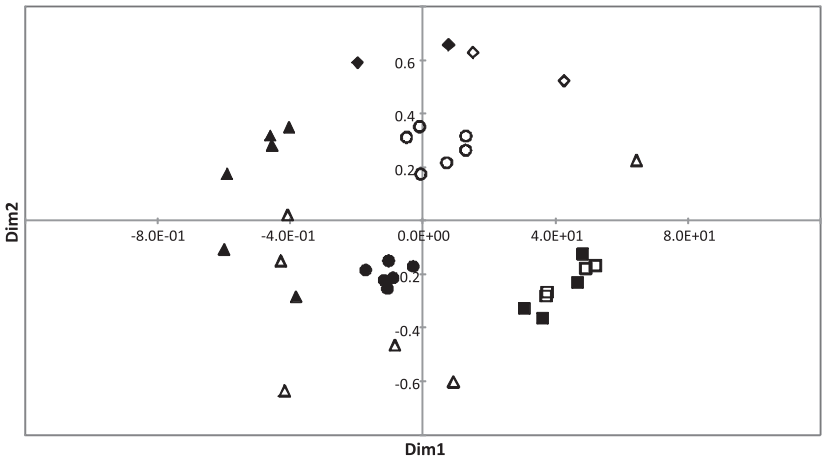

Fig. 3. Multidimensional Scaling of the presence/absence matrix deduced from the analysis of the DGGE gel derived from the amplification of the gene coding for 16S rRNA for all mangrove samples (black: samples from the Impacted plot, white: samples from the Reference plot, squares: sediment, triangles: root biofilm, circles: colonized biofilm, diamonds: water).

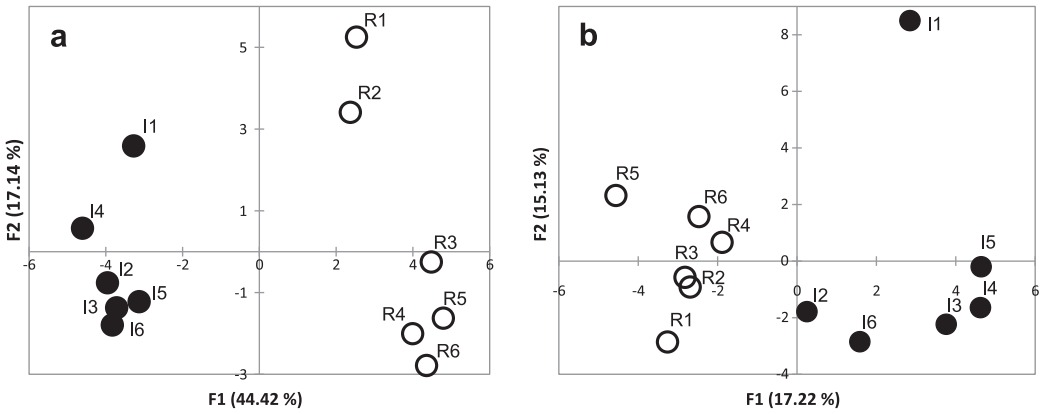

Fig. 4. Principal Component Analysis of the relative band intensity matrix deduced from the analysis of DGGE gels derived from the amplification of the gene coding for 16S rRNA (a) and 18S rRNA (b) for colonized biofilm samples (black circles: samples from the impacted plot I, white circles: samples from the Reference plot R).

as an accurate functional end-point for the characterization of phototrophic communities after such a short colonization period.

The composition of the prokaryotic community of colonized biofilms was homogeneous between replicates, while natural root biofilms displayed a more heterogeneous composition. Moreover the differences in composition between the two sampling plots appeared more clearly with colonized biofilms, than with natural samples. The values of species richness and diversity (Shannon index) of bacterial communities in colonized biofilms were intermediate between those found for the different natural communities (higher than water and root biofilm communities, lower than sediment communities). The diversity pattern of recent prokaryotic communities taken from the artificial substrates provided a reliable image of the pattern of mature natural communities, as it occupies a central intermediate position in the molecular diversity revealed using DGGE. As bacterial communities are known to be the earliest colonizers (Chan et al., 2003), these values probably indicate good development of the bacterial community. On the other hand, the eukaryotic community as a later colonizer, was assumed to be less developed. For this community developed on artificial substrates, mean diversity and richness values were high (24.2 and 2.7 respectively) and in the same range as those (mean values of 23 and 2.8 respectively) observed for fully-developed biofilms in freshwater ecosystems (Dorigo et al., 2010; Villeneuve et al., 2010). However, to our knowledge, no such values are yet available for eukaryotic communities from biofilms in tropical mangrove ecosystems.

Finally, the colonization device, involving glass plate surfaces, gave an accurate microbial score. Even if this device still needs to be tested on more shore areas before it can be generalized, this simple system looks promising as a way of revealing the richness and diversity of bacterial communities in tidal mangroves. Eukaryotic community diversity also seemed to be well assessed, but it too needs further evaluation. From a functional point of view, it looks as if a longer colonization time is required, but unfortunately the tidal rhythm does not allow for longer colonization periods without encountering daytime drought. Long exposure to dry conditions during tidal emersion may be hugely selective; masking any selection pressure linked to pollution, and can lead to slow community development. An alternative might be to use permanent pools located within the mangrove in order to permit longer incubation of the artificial substrates and to obtain mature biofilms well fitted for functional assessment of communities.

4.2. The microbial pattern along the trophic gradient

In the Chirongui bay mangrove, Lambs et al. (2011) and Herteman et al. (2011) first reported results at the vegetation and crab community scale, showing that their growth was boosted as a result of changes in the nutrient load in the mangrove sediments. However, analyses and observations of mangrove ecosystems as a whole are necessary to evaluate their bioremediation capacities as vegetative buffers against anthropogenic N-loading in coastal waters. The major aim of the work reported here was to assess the changes in microbial communities that may be attributable to changes in the nutrient load in the mangrove sediments. The effluent–receiving plot is characterized by having a greater nutrient load than the reference plot, particularly in the upper sediment layers for N forms, and in the deeper layers for P. As P and C levels, especially bioavailable forms, estimated respectively from the sediment upper layer and from the pre-treated effluent, were not very different from one plot to the other, these nutrients may not play a major role in the differential shaping of microbial communities in the two plots. The microbial communities observed in this study corresponded mainly to the surface oxic zone, and can therefore be expected to be more influenced by variations in N forms, while the other physico-chemical parameters did not exhibit major variations, and are typical of a seawater-dominated environment (Saenger, 2002).

4.3. Variations of the phototrophic community

The phototrophic microbial community was assessed on colonized biofilms, and clear differences of composition were detected between the impacted and reference plots. A higher contribution of green algae and diatoms characterized the photosynthetic potential of the phototrophic assemblage in the impacted plot. In the sediments of tropical mangrove forests, Farooq and Siddiqui (2011) reported a rapid decrease within the first few centimeters in the abundance of the microphytobenthic community, which may be directly related to changes in nutrient concentrations in addition to the light gradient.

No difference was found between the two plots with regard to diversity indices assessed by molecular fingerprinting (species richness and the Shannon index). Variations in nutrients did not seem to modify the number of eukaryotic DGGE-units in biofilms. However, a clear composition difference was observed between the eukaryotic assemblages of the two plots. As Villeneuve et al. (2010) pointed out, minor environmental changes that do not affect diversity can still result in modifications in the composition of community assemblages.

Within this community, diatoms seem to play an important role in the impacted plot, as shown by phytoPAM results. Diatoms are known to play a central role in coastal food webs, and they are routinely used to indicate the trophic status of rivers (Berthon et al.,

2011). In many aquatic ecosystems, nutrient composition is closely linked to the composition of the diatom community: for instance, within the Colne estuary in temperate England, ammonium concentrations have been shown to be important in determining the diatom species composition (Underwood et al., 1998), whereas in tropical Jamaica, phosphate and organic nitrogen were the main drivers (Podzorski, 1984). Dham et al. (2005) were able to link seasonal changes in the uptake of nitrogenous nutrients with the plankton community composition in mangrove waters. Pennate diatoms and flagellates were dominant during periods of high nitrate uptake, and centric diatoms and blue-green algae during those of high ammonium uptake. More recently, Chen et al. (2010) investigated the effect of environmental changes on mangrove diatoms, and revealed species associations that were closely linked to nitrogen and phosphate resources. Wachnicka et al. (2010) studied the distribution of diatoms in diverse subtropical habitat types in freshwater, mangrove, and marine locations, and assessed their compositions. In a given type of location, epiphytic, planktonic, and sediment assemblages were compositionally similar, implying a high degree of mixing along the shallow, tidal, and storm-prone coast. It was therefore hypothesized that a nutrient-linked assemblage was found in our impacted plot. These findings suggest that the phototrophic community, and diatoms in particular, could be used as sentinels of environmental changes in such mangrove ecosystems.

4.4. Variations of the prokaryotic community

We observed differences in the structure of the prokaryotic microbial community. The impacted plot, exposed to pre-treated wastewater discharges, contained a significantly higher density of bacterial cells, and this was mainly due to the abundance of the active bacteria fraction (HNA bacteria). They formed the dominant population in all samples (between 49% and 85% of the total bacterial community) with a higher average percentage in the impacted plot (75%). These results confirmed suggestions that nutrients could stimulate the growth of microbial populations in mangroves (Tam, 1998). However, it is also necessary to find out whether this increase may also result in efficient nutrient consumption and recycling. In an experimental study, Ramanathan et al. (2008) showed that in addition to the increase in total bacterial load, higher nutrient levels in sediments were also linked to higher abundances of N-2 fixing and nitrifying bacteria. The increase in bacterial density, combined with changes in the community composition, may therefore play a significant role in the fate of nutrients in our mangrove ecosystem.

Focusing on the colonized biofilms, a marked differentiation was observed between the microbial assemblages of the two plots. As shown by Peixoto et al. (2011), the composition of bacterial communities might be severly impacted by major environmental stressors, such as nutrients which may play a key role in shaping the community. This had previously been observed in mangrove sediments by Amano et al. (2011), who highlighted the presence of anammox and denitrification activities, although the contribution of anammox was less significant. Cao et al. (2011) revealed the presence of the *amoA* gene in polluted estuarine mangrove sediments, where high levels of nutrients and metals were recorded as a result of wastewater discharge from nearby rivers.

The high level of HNA bacteria, together with a specific community pattern in the impacted plot, both suggest the potential presence of a specific community particularly involved in nutrient consumption and transformation. However, our fingerprinting approach does not provide information about the identity of the species represented by differential DGGE-bands. Barcoding combined with high-throughput sequencing might offer a clearer understanding of the community assemblages by revealing the identity of species present in the different plots (dos Santos et al., 2011; Cleary et al., 2012). The specific bacterial populations involved and their role in the aquatic system should be determined by means of further studies of functional groups, such as N-Cycle bacteria for example (Santoro, 2010).

5. Conclusion

Our results, obtained using a simple and efficient colonization device for microbial communities, give a preliminary insight regarding the impacts of pre-treated wastewater effluents on microbial communities in a mangrove. In particular, a denser bacterial community exhibiting a specific assemblage that may be able to enhance nutrient recycling and consumption, have been observed in the impacted plot. However, further information about these systems is required. Data mining remains necessary, and should focus essentially on the diversity, structure and function of microbial communities in mangroves, which have so far received little investigation. Even less is known about their temporal and spatial dynamics. Finally, deeper insight into the functional diversity of these prokaryotic and eukaryotic communities is particularly necessary in order to obtain a clearer understanding of their ability to transform the effluents from the pre-treatment device, especially in the case of prokaryotic communities active in nitrogen cycling. More broadly, these results emphasize the importance of protecting mangrove ecosystems in order to enhance their buffer role against eutrophication of adjacent coastal marine waters.

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