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DISTRIBUTION OF PROKARYOTIC ORGANISMS IN A TROPICAL ESTUARY INFLUENCED BY SUGAR CANE AGRICULTURE IN NORTHEAST BRAZIL

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ABSTRACT

In a joint Brazilian-German case study, distribution patterns of microorganisms were compared with environmental variables in the tropical coastal Manguaba lagoon in northeast Brazil, which is situated downstream of several sugar cane processing plants . 16S rDNA and 16S rRNA single strand conformation polymorphism (SSCP) gene fingerprinting were used to follow the composition and distribution of microorganisms throughout the salinity gradient of the lagoon. Potentially abundant microorganisms were identified by sequencing representative SSCP bands. It could be demonstrated that the distribution of microbes was in close relation to the physico-chemical environmental settings and followed a common scheme. In the in- and outlet areas of the lagoon rather transient microbial communities were found, whereas in the central part a stable, diverse community was encountered, that due to the long residence time of the water, had ample time for development and adaptation.

Key words: tropical lagoon; estuarine gradient; microbial diversity; sugar cane

INTRODUCTION

In the mid-seventies, Brazil implemented the "Pro-Alcohol Programme" to boost the production of ethanol as an alternative energy source (23). Meanwhile, the production of agro-fuels has become a prime issue since the worldwide demand for alternative energy sources increased (1). More than 60 % of Brazil's sugar cane production is sustained by the State of São Paulo in the Southeast, and about 20 % along the coastal zone of the Northeast in the the States of Alagoas, Pernambuco and Sergipe.

Sugar-cane monoculture practices induce environmental impacts, as they involve the application of fertilizers and biocides, alter the composition and water retention capacity of soils, enhance land erosion and affect the quality and balance of groundwater (13). The materials and associated pollutants are introduced into aquatic systems by diffuse wash-out from the drainage basin and point- source emissions from industrial processing plants into rivers. The emission of sooth particles and PAH´s to the atmosphere from crop burning adds to environmental problems.

Most studies in Brazil addressing these problems focused

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on the limnic system, investigating the alterations of the biological oxygen demand, generated by the introduction of an waste product of sugar cane processing, the nutrient water quality, the impacts upon the sustenance of commercially relevant organisms, as well as air pollution effects (4, 6). However, the degree to which the multiple impacts from sugar-cane practices affect Brazilian estuaries and coastal waters has as yet to be discerned. Impacts on limnic and estuarine systems may differ considerably, as land-borne materials and associated pollutants undergo complex physico-chemical particle-water reactions during estuarine mixing due to changes of pH, salinity, biological production, and degradation processes (5, 15, 22).

Microbial communities play a key role in the transformation of inorganic and organic constituents, including pollutants. The microbial utilization of dissolved and particulate biogenic matter and a variety of pollutants is governed by the size of the molecules and particles, the nature of particle coatings and the reactivity and age of the materials (3). The composition and activity of microbial communities also differs between limnic, brackish and marine systems, as well as the composition of allochthonous material input and the systems trophic state itself (11). Tropical estuarine-coastal lagoons characterized by a high degree of enclosure and residence time of water, efficiently retain and transform river-borne and autochthonous produced matter and exhibit large spatial variations of microbial communities (12). When affected by multiple pollutant sources they serve as ideal sites for studies related to alterations of the composition and activity responses of microbial communities. The state of Alagoas provides an excellent model to investigate effects of monocultural land-use on estuarine transport patterns. This area is exemplary for the coastal region in the northeast of Brazil, were lagoons connect terrestrial and marine aquatic systems.

This study is part of the joint Brasilian / German Polcamar Project, which focuses on transport and impact of pollutants from sugar-cane monoculture to the coastal sea. It addresses the distributional patterns of microbial communities in its relation to changes of the physical-chemical environment along estuarine gradients of the tropical Manguaba lagoon system. By employing molecular biological approaches it attempts to describe and map the potentially abundant microbial community of this specific tropical estuarine system.

MATERIAL AND METHODS

Study site

The estuarine lagoon system of Mundaú-Manguaba, state of Alagoas, NE-Brazil (latitude 9.58° and 9.77°S and longitude 35.73° and 35.97°W) corresponds to a shallow (in average 2 m) choked lagoon and comprises three main sub systems: one being Mundaú lagoon (A= 24 km^2) towards the north, Manguaba lagoon (A= 43 km^2) towards the south and the mangrove dominated canal system (A= 12 km^2), which links both lagoons with the sea. The system is affected by its mesotides up to 2.7 m but 85% of the tidal energy dissipates within the canals which results in an average tidal range of 0.20 m in Mundaú and 0.03 m in Manguaba. The longest average water residence time is attained with up to 6 weeks in Manguaba (10). The climate of the system is tropical, semi-humid with well defined dry (October - March) and rainy seasons (May-August). The average annual rainfall is around 1,600 mm. The main rivers are the Paraibo do Meio in the upper compartment and the smaller Sumauma River in the lower compartment of Manguaba (Fig 1). The total daily average discharge freshwater is 28 m^3 sec^{-1}(16). The basins of both rivers are almost entirely covered by sugar cane monocultures and several processing plants use the rivers for extracting and discharging process water.

Sampling

Sampling was performed in March 2007 in the Manguaba lagoon system along a salinity gradient between 0 and 24 PSU between the outflow of the Sumauma river in the lower lagoon and the outlet of the lagoon into the Atlantic Ocean. The sampling stations are displayed in Fig. 1. Surface water samples for physico-chemical parameters were collected from the boat directly into 2 l bottles and processed further in the

laboratory. Samples for DOC measurements were filtered onto precombusted GFF filters (Whatman) and the filtrates were acididfied to pH 2 with 85 % H_3PO_4 and kept frozen until analysis. Samples for nucleic acid extraction were sampled directly from the surface water into a syringe, filtered immediately onto Nuclepore filters (pore size 0.2 µm) and cooled on ice. After return to the laboratory they were stored frozen at –20°C for later analysis.

Temperature, conductivity and oxygen were measured with WTW probes (WTW GmbH, Germany) and colorimetric nutrient analyses (NO_3, NO_2, NH_4, PO_4) where performed after filtration according to Grasshoff (9). Chlorophyll *a* and total suspended solids (TSS) were analyzed according to Strickland et al. (20). The DOC concentrations were measured using a high temperature combustion analyzer (Shimadzu TOC 5050) with a Platinum catalyst at 680°C. Canonical Correspondence Analyses (CCA) (21) were performed for for Salinity, Secci depth and DOC content date.

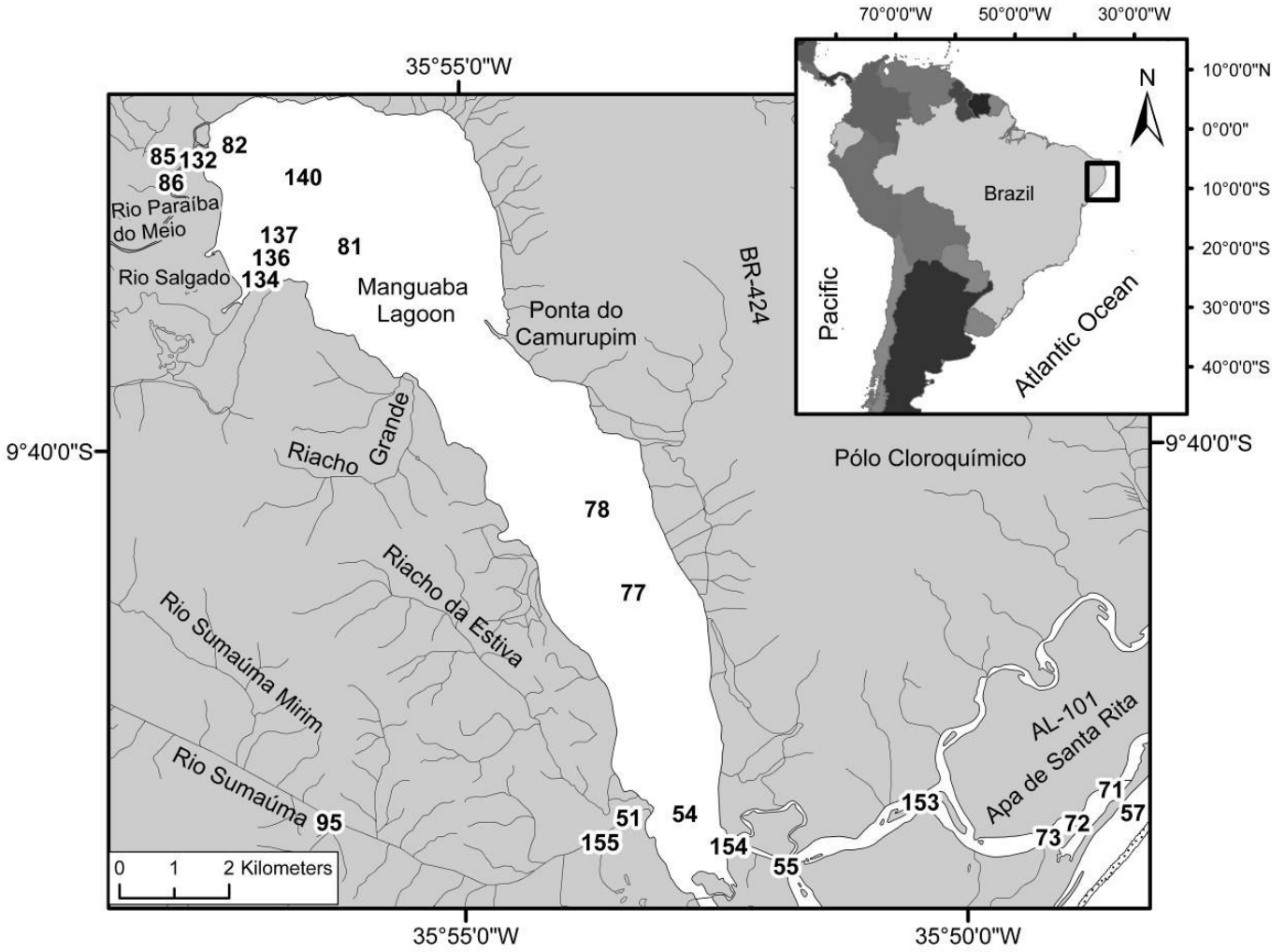

Figure 1. Sampling positions in the Manguaba lagoon as well as the supporting rivers and connected inlet canals. Sample positions 90 and 92 are not displayed; these samples were taken in drainage canals northeast of the lagoon. The position of the state Alagoas in Brazil is displayed in the right upper corner.

Nucleic acid extraction and ribosomal complementary DNA (rcDNA) synthesis

Nucleic acid extraction and quantification from the frozen filters was performed by parallel extraction of RNA and DNA using a phenol extraction protocol described by Weinbauer et al. (25) Prior to RT-PCR, RNA extracts were purified from DNA by incubation with DNase I (DNA-free-Kit, Ambion) for 30 min at 37°C and their concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). To retrieve 16S rcDNA 200 ng of template RNA were reverse transcribed at 42°C using the iScript cDNA synthesis kit (Bio-Rad). In addition to hexamers provided in the kit, the universal reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3') (14) was also applied. In each reverse transcription reaction, some RNA samples used as controls in the PCR were not supplemented with reverse transcriptase, in order to rule out DNA contamination.

16S rRNA fingerprint analysis

16S rcDNA and 16S rRNA genes were analyzed by single strand conformation polymorphism (SSCP). Bacterial Com-primers (amplifying positions 519 to 926 of *E. coli* numbering of 16S rRNA gene) (19) were used for gene amplification. Thermocycling started with an initial denaturation for 5 min at 94°C. A total of 25 cycles (30 s at 94°C, 30 s at 55°C, 2 min at 72°C) were followed by a final elongation step of 10 min at 72°C. 16S rRNA genes were amplified analogously with a total of 35 cycles. Generation and purification of single-stranded DNA (ssDNA) and SSCP analysis were performed according to Schwieger and Tebbe (19). Re-amplification of individual bands excised from the SSCP gels was performed as described by Pöhler et al.(18) PCR products were purified using the NucleoSpin® Extract 2 (Macherey & Nagel) as described by the manufacturer and were sequenced by Qiagen (Hilden, Germany). Forward and reverse sequences of all samples were checked for accuracy using the SeqMan software (DNASTAR).

Phylogenetic analyses

Phylogenetic affiliations of the partial 16S rRNA sequences were estimated using the basic local alignment search tool BLAST (2). The 16S rRNA gene sequences determined in this study were deposited in the GenBank database under accession numbers GU88510 to GU088529.

RESULTS

Environmental parameters

Means of environmental variables measured at specific sections within the different estuarine provinces coincided largely with the geographical division (Tab. 1) of the lagoon system, this was also affirmed by CCA statistic analyses: **(I)** the limnetic part of the estuarine gradient, the river itself and the areas close to the river mouth were characterized by turbid, low saline water with high concentrations of phosphate, nitrate and silicate (Tab. 1). This limnetic section was sampled in drainage channels and the rivers Paraibo do Meio and Sumauma as suppliers to the lagoon. **(II)** The central Manguaba lagoon was a brackish water body with distinct endemic features. Due to the sedimentation of the riverine mineralic particles to the bottom of the lagoon, light penetrated far deeper into the water and promoted intensive phototrophic activity (Tab. 1). At a salinity of about 4 PSU chlorophyll *a* values were an order of magnitude higher than in the supplying rivers with associated lower values of inorganic nutrients and higher oxygen concentrations. Light microscopic inspection of the phytoplankton community showed a mixture of green algae, diatoms and colonial and filamentous cyanobacteria as dominant groups (data not shown). DOC concentrations showed a patchy distribution within the central Manguaba lagoon. **(III)** The outlet channels of the lagoon system are the connection to the Atlantic Ocean, where brackish lagoon and coastal marine waters mix. The mixing gradient was, however, not continuous, but strongly influenced by tides. Due to the generally lower DOC concentration of coastal ocean water, DOC concentrations in the channels were also lower, if compared to the central lagoon, as a result of mixing with coastal waters (Tab. 1). Chlorophyll *a* values were still high but lower oxygen concentrations pointed towards lower phototrophic activity and increased heterotrophic processes.

Table 1. Physical and chemical parameters of the sampling stations. (ND, not determined)

Station [Polca]	Latitude [S]	Longitude [W]	Temperatur [°C]	Conductivity [ms]	Salinity [PSU]	O_2 [µM]	Secchi depth [m]	Chlorophyll *a* [mg m^3]	Phaeopigments [mg m^3]	TSS [mg l^{-1}]	Nitrate [µM]	Nitrite [µM]	Ammonia [µM]	Phospate total [µM]	Phospate diss. [µM]	DOC [mg l^{-1}]
Paraibo do Maio river , the Sumauma river and drainage canals (I)																
Mean			29.09	0.21	0	150.53	0.00	3.91	5.09	65.42	15.23	1.13	2.03	3.44	1.60	9.86
standard deviation +/-			1.55	0.07	0	122.03	0.00	1.63	3.69	44.29	18.85	0.72	2.56	1.57	1.26	6.09
155	-9.73	-35.89	28.50	0.13	0	17.12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
095	-9.73	-35.94	28.70	0.14	0	254.41	ND	2.81	5.49	32.00	8.40	1.09	0.79	2.76	1.24	20.96
051	-9.73	-35.89	28.70	0.15	0	34.25	0.00	5.82	0.00	18.00	0.75	0.43	0.33	2.33	0.57	8.87
092	-9.57	-35.87	32.80	0.18	0	319.59	ND	5.23	3.74	145.50	ND	ND	ND	ND	ND	3.03
090	-9.55	-35.85	27.70	0.20	0	296.35	ND	1.92	3.74	62.50	36.55	1.86	4.98	5.24	3.00	8.50
086	-9.62	-35.96	28.70	0.26	0	73.39	0.00	5.00	11.12	70.00	ND	ND	ND	ND	ND	11.25
085	-9.62	-35.96	29.00	0.29	0	72.10	0.00	2.69	6.47	64.50	ND	ND	ND	ND	ND	6.55
132	-9.62	-35.96	28.60	0.32	0	136.99	0.00	ND	ND	ND	ND	ND	ND	ND	ND	ND
Manguaba lagoon upper and lower compartment (II)																
Mean			29.88	8.55	4.25	180.29	0.48	34.89	86.90	38.50	13.43	0.61	2.82	4.07	1.08	7.40
Standard deviation +/-			0.45	2.29	1.36	51.14	0.20	14.03	36.68	12.67	14.22	0.29	1.90	3.00	0.70	1.58
082	-9.62	-35.96	30.30	5.99	3	137.08	0.40	24.39	59.61	47.50	42.90	1.14	6.06	8.81	1.43	4.38
140	-9.62	-35.94	30.30	6.31	3	171.93	0.50	37.73	88.53	41.00	7.42	0.64	2.41	2.19	1.19	8.99
081	-9.63	-35.93	29.50	6.65	3	244.50	0.5-0.8	ND	ND	ND	ND	ND	ND	ND	ND	ND
137	-9.63	-35.95	30.30	6.68	3	153.35	0.50	22.14	57.52	41.50	7.96	0.64	4.56	2.62	1.38	8.76
134	-9.64	-35.95	30.20	6.71	3	146.38	0.35	30.95	78.41	26.06	10.03	0.61	0.06	4.67	1.38	6.79
136	-9.63	-35.95	30.30	6.76	3	160.76	0.60	31.41	78.33	30.40	6.16	0.56	4.25	3.19	1.14	6.29
154	-9.73	-35.87	30.00	9.81	5	208.09	ND	28.80	71.74	25.17	8.71	0.24	1.60	1.81	0.33	ND
153	-9.73	-35.84	29.70	9.21	5	140.29	ND	41.03	105.70	41.14	0.97	0.37	1.60	1.76	0.29	ND
078	-9.68	-35.89	29.40	9.49	5	299.93	0.65	13.62	28.16	68.00	28.72	1.09	4.48	10.81	2.62	8.44
054	-9.73	-35.88	29.90	11.65	6	151.13	0.55	57.12	141.54	27.25	1.49	0.43	1.14	3.10	0.38	8.02
055	-9.74	-35.86	29.80	11.89	6	137.22	0.55	60.08	155.17	30.50	1.71	0.27	1.02	3.81	0.43	8.76
077	-9.69	-35.89	28.90	11.50	6	212.81	0.70	36.57	91.24	45.00	31.64	0.72	3.79	2.05	1.33	6.14
inlet canal between Manguaba and the Atlantic ocean (III)																
Mean			29.10	27.60	15.75	110.52	0.83	29.64	72.21	38.51	22.62	0.55	4.82	3.63	0.46	5.48
Standard deviation +/-			1.24	3.21	1.50	82.24	0.21	13.96	35.97	43.21	17.25	0.26	2.61	3.03	0.11	2.19
057	-9.73	-35.81	30.20	19.11	10	0.00	ND	58.43	143.01	46.00	2.24	0.40	2.02	4.38	0.33	8.59
073	-9.73	-35.82	28.90	21.20	12	130.42	0.85	24.39	60.30	28.20	33.67	0.66	8.25	5.14	0.52	4.43
072	-9.73	-35.82	28.80	29.80	17	146.87	0.80	21.67	52.02	45.12	26.47	0.69	4.79	2.90	0.62	3.57
071	-9.72	-35.81	28.50	40.30	24	164.80	ND	14.05	33.49	34.71	28.11	0.45	4.22	2.10	0.38	5.32

Microbial distribution and identification

For the whole estuarine gradient 20 microorganisms could be allocated to either *Proteobacteri*a, *Cyanobacteria* , *Bacteroidetes*, or *Actinobacteria* (Fig. 2). The quality of this allocation was dependent on the match between extracted and deposited sequences and in the range from 91 to 100 % (Fig. 2). The distribution patterns of prokaryotic microorganisms in general followed the scheme of environmental parameters described above: **(I)** In the limnic part of the estuarine gradient, the river itself and the areas close to the river outlet different *Beta-*, *Epsilon-*, and *Gammaproteobacteri*a were abundant in the fingerprint analyses. **(II)** Several of the organisms detected in the limnic part of the lagoon were also found in the central lagoon with its brackish water body, but in general the detected microbial diversity increased with more organisms belonging to the *Gammaproteobacteria* - especially *Acinetobacter* - *Cyanobacteria*, *Bacteriodetes*, and *Actinobacteria*. *Cyanobacteria* were mostly represented by *Synechococcu*s spp. The fraction of *Betaproteobacteria* decreased and was nearly undetectable on rRNA level. No *Epsilonproteobacteria* were detected anymore. **(III)** The outlet channels of the lagoon system were characterized by microbial assemblages comparable to the ones of the central lagoon system. However, no 16S rRNA of *Betaproteobacteria* could be detected in this system anymore.

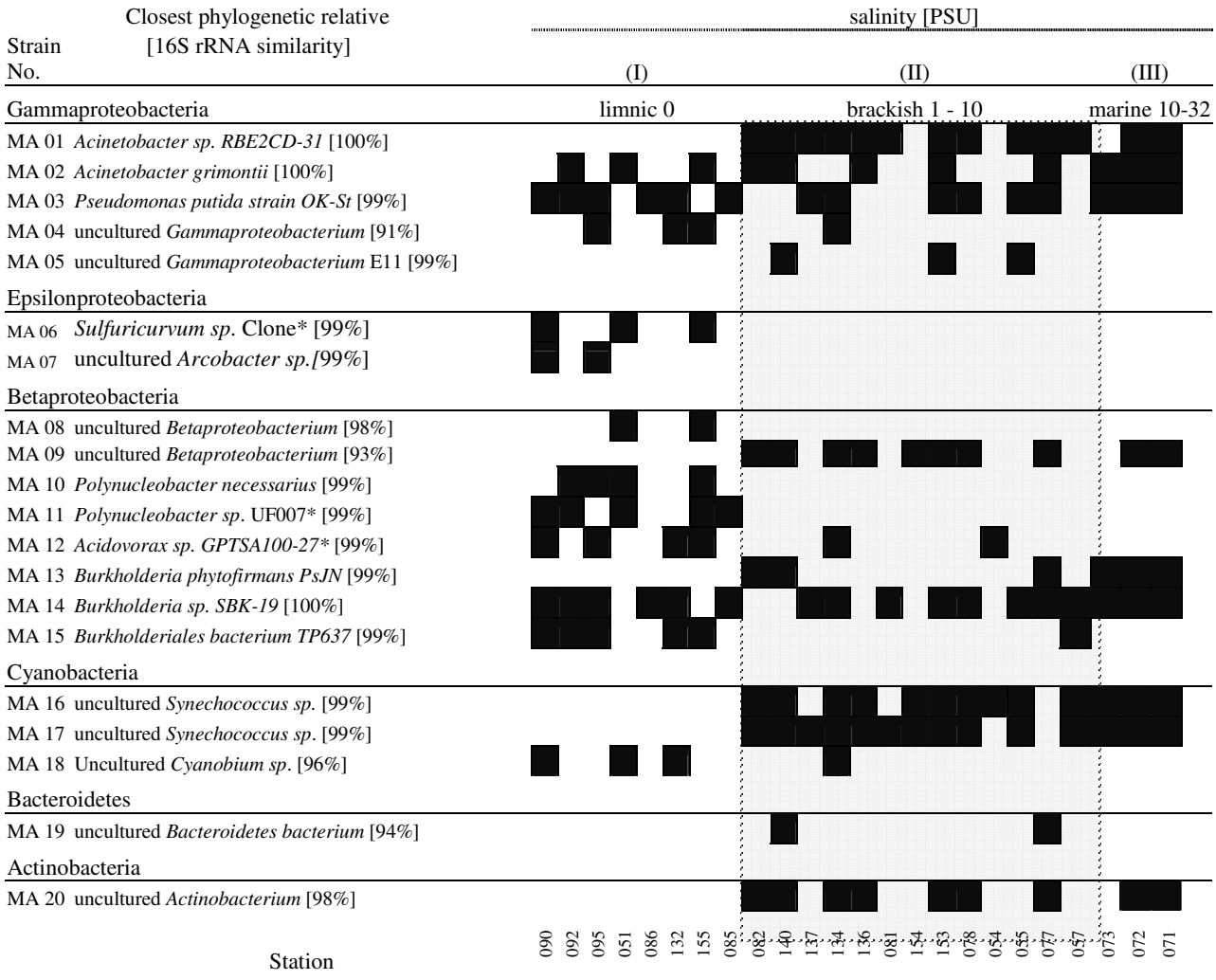

Figure 2. Horizontal distribution of identified taxa based on 16S rRNA gene or 16S rRNA SSCP fingerprinting. Bacteria identified on DNA as well as on RNA level are indicated in bold. Bacteria identified only on RNA level are marked by asterisks.

DISCUSSION

The results both from environmental measurements and analyses of the microbial communities classify the Manguaba lagoon as an estuary with three different subsystems with an overall coherence between environmental indicators and microbial abundance patterns:

(I) Limnic system

The first subsystem, comprising of rivers feeding the lagoon and regions at the river inlets, was characterized by physicochemical and biological features which were indicative for a non-steady state situation. In our study period large amounts of nutrients, organic matter, and microorganisms have quite recently been washed into the river systems along the cane fields. Although the rainfall in March 2007 with 169 mm/month was only slightly above the statistical average monthly precipitation for March, the rain was concentrated on a few extreme events in the range from 40 mm to 60 mm per day. This led to heavy local erosion of soil, organisms and elevated input of dissolved organic and inorganic compounds. High DOC values are supposed to derive from the same source as well as from direct input of organic constituents from the sugar processing factories. *In situ* production of DOC by algae was unlikely due to the fact that chlorophyll *a* and pigment values were low as an effect of light limitation in the highly turbid waters. All this was reflected in the chemical and microbial composition within the river water with a bacterial community typical for soils and freshwater (Fig. 2). Especially *Betaproteobacteria* related 16S rRNA bands MA 15 or MA 8 were indicative for soils or associated with terrestrial plants roots, respectively (7, 24). MA 14 related sequences belonging to *Burkholderia* were isolated from tropical soils which had been influenced by fires (17). The species *Burkholderia phytofirmas* PsJN, highly related to sequence MA 13, was described in a study on endophytic bacteria in sugar cane which show beneficial effects on plant growth, but may also be associated with putative opportunistic human pathogenic bacteria (16). Other sequences found in this environment (MA 11, 10, 12) were related to typical members of bacterial fresh water plankton like *Polynucleobacter*-, or *Acidovorax*-related sequences. The potential abundance of *Betaproteobacteria* in this part of the gradient was expected, as this group is known as a major member of the limnic compartment of estuarine systems (6, 8). *Gammaproteobacteria* as the next abundant group is an ubiquitous class of bacteria found in most known habitats and so their presence could as well be anticipated.

To summarize, high concentrations of dissolved organic carbon (DOC) and inorganic nutrients (Tab. 1) fostered potential heterotrophic organisms and activity. Photoautotrophic processes and their contribution to DOC production were low due to high turbidity and therefore the system can be considered to be heterotrophic. Both substrate and decomposers were probably to a large degree imported from soil and due to the short residence time in the aquatic environment can not be assumed to be in a decomposition equilibrium.

(II) Brackish system

The above described scenario changed, once the water entered the second subsystem – the central lagoon. The turnover time of water in the central lagoon is in the range of 36 days (10) and therefore supports many generation times of unicellular organisms which form a self sustained microbial system. As the introduced mineralic matter quickly sank out of the water at the river inlets due to a reduction of turbulence in the open lagoon, light penetrated deeper into the water and enabled photoautotropic production. This in turn provided organic matter which can be decomposed and mineralized right away. An autogenic balanced production-decomposition system was established in the lagoon, where the microbes were probably more adapted to intrinsic conditions than to quantity and quality of external input variables. This was reflected in chlorophyll increase, nutrient decrease (Tab. 1) and as well in the community composition, with different autotrophic cyanobacteria, mostly related to the picocyanobacterium *Synechococcus*, and several heterotrophs (Fig. 2). Elevated

concentrations of ammonia indicated a high turnover of organic substrates and a functioning cycle of matter between autotrophic and heterotrophic agents. High productivity in lakes and lagoons usually means an increased carbon flux towards the sediments with associated oxygen deficiencies in the bottom water in spite of a well oxygenated surface layer. In general, the higher relative abundance of *Gammproteobacteria*-related sequences corresponded to the findings of Bernhard et al. (6) who also studied the distribution of 16S rRNA genes in estuarine systems. Within this group, *Acinetobacter* was abundant in the brackish system. From these, phylogenetic relatives of SSCP bands MA 1 or MA 4 have been found to degrade persistent organic pollutants. Thus, within the natural microbial assemblage of the lagoon a high potential for resistance against xenobiotic substances or even for detoxification could exist.

Introduced substances will remain here for a considerable time span and will meet successive environmental conditions of extreme diversity. Water and dissolved substances are transported repeatedly through considerable environmental gradients, which allows specialized organisms to attack these substrates. In these diverse environments bacteria can potentially be found with a high variety of different metabolic pathways and the ability to withstand and degrade complex organic substances. In the central lagoon we found a system which had the potential, both in environmental background conditions and in terms of microbial diversity, to efficiently modify introduced substances. Considering the presence of specialized bacteria, which were able to cope with organic pollutants, this points towards a certain decontamination potential.

(III) Outlet channels

At the outlet channels turbidity as well as oxygen concentration was lower than in the brackish water (Table 2). The banks of these channels were grown over with mangrove stands, removing a high amount of particulate matter from the water. In addition to the mixing with clear water from the coastal ocean this may lead to a further reduction of turbidity as compared to the central lagoon and an increase in nitrate and ammonia values, whereas orthophosphate seemed to be kept within the sediments. No betaproteobacteria were detectable on 16S rRNA level anymore, indicating that this limnic group was inactive at more saline conditions. The remaining detected microbial diversity was comparable to the brackish system; however, increased turbulence, reduced water residence times in specific environments and the tidal mixing generated a strong salinity gradient (ranging from 10 to 24 PSU). In contrary to the central lagoon, these conditions demanded high adaptation efforts within a short time span, probably resulting in a decrease of decontamination efficiency.

The general conclusion of this study is, that the Manguaba lagoon in its present condition has a diverse and well adapted microbial community. A system like this is generally adapted to cope with additional external loads of natural substances, as it operates on an elevated decompositional level due to the high internal substrate turnover. In Lagoa Manguaba this is based on the morphology of the lagoon and the long residence time of water as well as on the presence of a variety of adapted microbial organisms. Drastic difference in functional properties of this system could probably be expected if the water residence times are changed by technical measures or major changes in the drainage patterns.

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