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Padrões espaciais e temporais da composição e atividade do bacterioplâncton no estuário da Lagoa dos Patos (RS, Brasil)

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Dedico aos procariotos que, tão ínfimos, mantém o equilíbrio dinâmico daTerra.

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LISTA DE ABREVIATURAS E SIGLAS

ACC/CCA - “canonical correspondence analysis” / análise de correspondência canônica

ANOSIM - “analysis of similarity”

CCB/BCC - composição da comunidade bacteriana / “bacterial community composition”

DGGE - “denaturing gradient gel electrophoresis”

DNA - “deoxyribonucleic acid”

ENOS/ENSO - El Niño oscilação sul / “El Niño southern oscillation”

GC - guanina e citosina

NPMANOVA- “Non-parametric multivariate analysis of variance”

PCR - “polymerase chain reaction”

PFCB/CLPP - perfil fisiológico da comunidade bacteriana / “community level physiological profiles”

SAPM/SBPM - substâncias de alto peso molecular / substâncias de baixo peso molecular

SOI - “Southern Oscillation Index”

Tm - “temperature of melting”

TTGE - “temporal temperature gradient gel electrophoresis”

Vr - valores de referência

16s rRNA - gene que codifica o RNA (“ribonucleic acid”) ribossomal que compõe a sub-unidade menor do ribossomo bacteriano

NH_4^+ / NO_2^- / NO_3^- / PO_4^{-3} - íon amônio / nitrito / nitrato / fosfato.

RESUMO

A composição (CCB) e a atividade (perfil fisiológico - PFCB) da comunidade bacteriana foram investigadas no estuário da Lagoa dos Patos e região costeira adjacente através de amostragem tipo-Lagrangiana, Euleriana e ao longo de um transecto para responder três perguntas: i) existe um padrão de recorrência (estabilidade) da CCB e PFCB em diferentes faixas de salinidade? ii) a CCB e PFCB respondem a diferentes escalas temporais e espaciais, inclusive a fenômenos climáticos globais que afetam a hidrodinâmica do estuário, tais como o “El Niño Southern Oscillation” (ENSO) ? iii) que fatores, além da salinidade, afetam a CCB e a PFCB? A CCB e a PFCB estiveram associadas à hidrodinâmica do estuário, tanto em escalas curtas (entrada de cunha salina), médias (sazonal) e largas (ENSO). Isto porquê a hidrodinâmica condiciona a variabilidade da salinidade e secundariamente o seston, nutrientes inorgânicos dissolvidos e substratos orgânicos, que afetam as bactérias. A CCB foi primariamente estruturada pela salinidade, seguindo o padrão normalmente encontrado na literatura para estuários, com comunidades características de água salgada e doce. Já a PFCB teve maior influência da quantidade de nutrientes e substratos e de maneira indireta da salinidade. De maneira geral, a atividade bacteriana foi menor em águas salgadas mais oligotróficas. Entretanto, grande atividade bacteriana foi observada em água salgada rica em nutrientes que penetrava no estuário. A maior concentração de nutrientes na água salgada pode ter sido resultado de ressuspensão de sedimento, ou ingresso de água costeira previamente enriquecida com água estuarina..

PALAVRAS-CHAVE: Temporal Temperature Gradient Gel Electrophoresis / Ecoplate / nutrientes / bactéria / hidrodinâmica / ENSO

ABSTRACT

The composition (BCC) and activity (community level physiological profiles, CLPP) of the bacterial community were investigated in the Patos Lagoon estuary and adjacent coastal region through Lagrangian-like, Eulerian and transect samplings in order to answer three questions: i) is there a pattern of recurrence (stability) of the BCC and CLPP in different salinity ranges? ii) does the BCC and CLPP respond to different temporal and spatial scales, including global climate phenomena that affect the estuary hydrodynamics like El Niño Southern Oscillation (ENSO)? iii) which factors apart from salinity affect the BCC and CLPP? The BCC and CLPP were associated to the estuary hydrodynamics, at short- (salt wedge entrance), meso- (seasonal) and large-scales (ENSO). This is because the hydrodynamics conditions the variability of salinity and secondarily the seston, dissolved inorganic nutrients and organic substrates, which in turn affect bacteria. The BCC was primarily structured by salinity, following the pattern commonly found in the literature for estuaries, with characteristic fresh- and saltwater communities. The CLPP had higher influence of the amount of nutrients and substrates and indirectly of salinity. In general the bacterial activity was lower in oligotrophic, saltier waters. However, high bacterial activity was observed in nutrient-rich saltwater that entered the estuary. The higher concentration of nutrients in saltwater is likely the result of ressuspension of the sediment or ingress of coastal water previously enriched with estuarine water.

KEY-WORDS: Temporal Temperature Gradient Gel Electrophoresis / Ecoplate / nutrients / bacteria / hydrodynamics / ENSO

1. INTRODUÇÃO

1.1 Justificativas

As bactérias exercem um papel fundamental em todos os ecossistemas, pois são responsáveis pela maior parte das respirações aeróbica e anaeróbica, da decomposição de elementos e regeneração de nutrientes (Cole 1999). Além disso, podem regular a concentração e mobilização de nutrientes na coluna d'água (Kirchman 1994), competindo com o fitoplâncton pelos mesmos em diversas situações (Cotner & Biddanda 2002). As bactérias representam também um importante elo na transferência de matéria orgânica dissolvida e energia para níveis tróficos superiores (Pomeroy 1974, Azam et al. 1983). Os processos bacterianos, entretanto, dependem em grande parte da composição taxonômica e riqueza de espécies (Bell et al. 2005). Desta forma, avaliar as variáveis que influenciam a diversidade bacteriana é crucial para se entender melhor a dinâmica dos micro-organismos e de seus serviços ao ecossistema.

A salinidade tem sido reconhecida como o fator ambiental mais relevante na estruturação de comunidades[1] bacterianas (Lozupone & Knight 2007). A diferenciação filogenética entre bactérias de água doce e de água salgada deriva de sua história evolutiva, uma vez que os primeiros micro-organismos surgiram nos oceanos pré-cambrianos em condições de altas temperaturas e salinidade, tendo a colonização da água doce ocorrido somente mais tarde (Knauth 2005), após a consolidação das primeiras massas continentais, há aproximadamente três bilhões de anos (Rogers & Santosh 2003). Esta longa

1 - O termo comunidade é empregado como sinônimo de assembleia o qual, por definição, compõe um conjunto de organismos taxonomicamente relacionados e que ocupam o mesmo local ao mesmo tempo (Fauth *et al*. 1996). Este emprego é justificado pela sua ampla aceitação dentro da área da ecologia microbiana

evolução em ambientes contrastantes originou a atual marcante diferenciação filogenética entre bactérias de água doce e salgada (e.g. Wu *et al*. 2006).

Grande parte desta diferenciação filogenética deriva da capacidade de colonizar ambientes que apresentam níveis de estresse salinos diferentes. Praticamente todas as bactérias apresentam adaptações para lidar com mudanças externas de osmolaridade, entre estas se destacam a acumulação de solutos (K^+ e glutamato) em resposta a estresse hiperosmótico, ou a abertura de canais mecanossensitivos (que liberam água) em resposta a choque hiposmótico (Booth *et al*. 2008). Porém, estas adaptações atuam em níveis limitados de estresse salino e muitas bactérias não sobrevivem às mudanças abruptas de salinidade encontradas em estuários, por exemplo. Isto é comprovado pelo aumento do percentual de células com membranas citoplasmáticas danificadas ou despolarizadas nas zonas de mistura quando comparadas com locais com menor ou maior salinidade (Cottrell & Kirchman 2004).

Neste contexto os estuários, corpos d'água parcialmente rodeados por terra onde há a mistura de águas doce e marinha (Garrison 2010), tornam-se ambientes muito interessantes para o estudo da composição de comunidades bacterianas. Estes ambientes apresentam alta produtividade e diversidade biológicas (Garrison 2010) e promovem o encontro de comunidades de organismos de origens e composições distintas, com a possível mistura de organismos de origem continental e marinha (Trousselier et al. 2002), havendo a possibilidade do estabelecimento de uma comunidade tipicamente estuarina dependendo do tempo de residência da água (Crump et al. 2004). De maneira

geral, ao longo dos gradientes salinos nos estuários reconhece-se a alternância de dominância de β-, δ-, ε-Proteobacteria e Actinobacteria em água doce para a prevalência de α-Proteobacteria e γ-Proteobacteria em água salgada (Glöckner *et al*. 1999; Yokokawa *et al*. 2004; Campbell & Kirchman 2012). O grupo Proteobacteria compreende uma das maiores divisões de todos os procariotos e a maioria das bactérias Gram negativas, com diversas feições fenotípicas e fisiológicas. Estas incluem foto-, hetero- e quimiolitotrofia e parasitismo (Gupta 2000 e referências). O grupo Actinobacteria faz parte do grupo dos Actinomicetos, bactérias Gram positivas com alto percentual de GC no seu conteúdo genético e incluem representantes aeróbicos, anaeróbicos facultativos ou anaeróbicos; muitos gêneros são característicos de solos, com papel relevante na ciclagem nutrientes (Goodfellow 1983 e referências). A salinidade também parece modular a atividade bacteriana e o padrão geralmente encontrado de diminuição da atividade em águas mais salinas, conforme medido por vários métodos independentes, tais como a incorporação de leucina e timidina radiomarcadas (Trousselier et al. 2002, del Giorgio & Bouvier 2002), percentual de células com respiração ativa (CTC+), quantidade de bactérias com alta quantidade de DNA (high-DNA) (del Giorgio & Bouvier 2002) e atividade ectoenzimática (Cunha et al. 2000).

A hidrodinâmica da maioria dos estuários é dominada em alguma extensão por marés (Wells 1995), apresentando regimes de meso- (2-4 m) e macromaré (> 4m). Nestes ambientes, as marés determinam a distribuição de salinidade e sedimentos (Perillo 1995). No entanto, uma terceira classe de estuários não sofre grande influência de maré apresentando um regime de micromaré (< 2m) e a tendência de ser influenciada principalmente por ondas, vento (Perillo 1995)

e descarga de água doce (Luettich et al. 2002). Podem apresentar, portanto, grandes variações nas condições ambientais em várias escalas temporais, variando desde horas até escalas sazonais (Hartmann & Schettini 1991) ou eventos esporádicos, mas de grande efeito como os fenômenos climáticos de larga escala como o El Niño Oscilação Sul (ENOS ou ENSO, “El Niño Southern Oscillation”) (Garcia *et al*. 2004; Abreu *et al*. 2010).

Observa-se, no entanto, que a maioria dos modelos de dinâmica de condições ambientais tem sido desenvolvida para estuários temperados e dominados por marés no hemisfério norte, ao passo que estuários de micromaré e no hemisfério sul têm sido pouco estudados (Taljaard *et al*. 2009). Isto é um ponto crítico, uma vez que os estuários do hemisfério norte apresentam descargas anuais menos variáveis que estuários temperados e subtropicais do hemisfério sul (Braune 1985, Taljaard *et al*. 2009) e, portanto, estes últimos podem apresentar diferenças importantes na hidrodinâmica, que repercutem nos padrões dos organismos.

Dentre os estuários de micromaré do hemisfério sul, destaca-se o estuário da Lagoa dos Patos, que se localiza na região costeira do estado do Rio Grande do Sul (Brasil). Neste estuário, estudos sobre a abundância e atividade bacterianas foram realizados (Cesar e Abreu 2001), destacando-se os seguintes pontos: i) foi encontrada uma relação entre a abundância bacteriana e fosfato e ausência de relação significativa com clorofila, o que sugere que outras fontes de energia (macrófitas e efluentes urbanos) são mais importantes do que a produção primária do fitoplâncton para a produção bacteriana (Abreu et al. 1992, Abreu & Odebrecht 1995); ii) a biomassa bacteriana pode exceder a biomassa fitoplanctônica durante vários meses, com efeitos importantes

sobre a teia trófica do estuário (Abreu et al. 1992); iii) foi encontrada uma correlação positiva entre nitrito+nitrato e a abundância bacteriana, o que foi atribuído à covariância destes nutrientes com a salinidade, mas também à absorção direta de nitrito pelas bactérias, com implicações para competição com o fitoplâncton (Abreu et al. 1992); iv) não foi encontrada uma relação entre bactérias aderidas e seston, o que sugere que a re-suspensão não contribui significativamente para o aumento da abundância de bactérias aderidas na coluna d'água (Abreu et al. 1992); v) bactérias livres respondem mais à variações do fitoplâncton do que as aderidas, que apresentam abundância mais constante (Abreu et al. 1992); vi) a concentração de nutrientes inorgânicos (N e P) exercem forte influência sobre o crescimento das bactérias (Cesar 1997, Cesar & Abreu 1998). Foi constatado o papel central das bactérias na teia trófica do estuário da Lagoa dos Patos (Abreu et al. 1992), na decomposição de macrófitas (Anésio et al. 2003, Hickenbick et al. 2004) e sua participação na ciclagem de nutrientes neste ecossistema (Cesar 2002, Hickenbick 2002).

No entanto, importantes questões a respeito das comunidades bacterianas ainda não foram abordadas, especialmente sobre sua diversidade em diferentes escalas temporais e espaciais como, por exemplo, i) se existe um padrão de recorrência (estabilidade) de espécies de bactérias e suas atividades em diferentes faixas de salinidade? ii) se a composição das comunidades de bactérias e sua atividade respondem a diferentes escalas temporais e espaciais, inclusive a fenômenos climáticos globais que afetam a hidrodinâmica do estuário, tais como o ENSO? iii) que outros fatores, além da salinidade, afetam a composição da comunidade bacteriana e suas atividades?

O presente trabalho pretende contribuir para a compreensão destas questões tão importantes e que afetam a ecologia do estuário. Como contribuição prática, o desenvolvimento desta tese permitiu a implementação de duas técnicas: o “temporal temperature gradient gel electrophoresis” (TTGE), uma técnica de biologia molecular utilizada para comparar a composição de ribotipos de diferentes amostras (“fingerprinting”) e o ensaio de capacidade de degradação de compostos orgânicos através de placas comerciais (Ecoplate™ Biolog). Estas técnicas permitiram refinar os estudos de diversidade e atividade bacterianas que vem sendo conduzidos no Laboratório de Fitoplâncton e Microorganismos Marinhos do Instituto de Oceanografia da FURG (mais detalhes dos métodos e suas limitações serão apresentados nos itens Material e Métodos e Discussão).

1.2 Hipóteses

Com relação a estas questões foram estabelecidas as seguintes hipóteses de trabalho:

i) Existem comunidades bacterianas específicas para diferentes faixas de salinidade;

ii) As comunidades bacterianas variam espacial- e temporalmente em função da hidrologia do estuário da Lagoa dos Patos podendo, inclusive, sofrer efeitos de fenômenos climáticos globais que sabidamente influenciam a hidrologia deste estuário como o ENSO.

iii) outros fatores como a disponibilidade de nutrientes e substratos afetam a composição e atividade das comunidades em diferentes faixas de salinidade.

1.3 Objetivos

1.3.1 Objetivo geral:

Investigar padrões espaciais e temporais da composição filogenética e da atividade da comunidade bacteriana no estuário da Lagoa dos Patos

1.3.2 Objetivos específicos:

1. Investigar a estabilidade da composição filogenética e da atividade da comunidade bacteriana utilizando três faixas de salinidade (limnética-oligohalina: 0-1, mesohalina: 14-16 e polihalina: 28-31) como unidades amostrais durante um ano;
2. Determinar se há variação da composição filogenética e da atividade da comunidade bacteriana em resposta a variáveis ambientais alteradas pelo fenômeno ENSO (transição de um evento de El Niño para La Niña);
3. Investigar a variação espacial em larga escala (km) e temporal de curta escala (dias) da composição filogenética e da atividade da comunidade bacteriana no estuário da Lagoa dos Patos, contemplando amostras superficiais e de fundo.

2. MATERIAL E MÉTODOS

2.1 Área de estudo

A Lagoa dos Patos (figura 1) é a maior lagoa costeira do tipo estrangulada do mundo, com aproximadamente 10^4 km^2 e parte de uma bacia hidrográfica de 2×10^5 km^2 (Kjervfe 1986). Apresenta uma descarga média de água doce de aproximadamente 2.000 m^3 s^{-1}, podendo chegar a 30.000 m^3 s^{-1} (Marques et al. 2009).

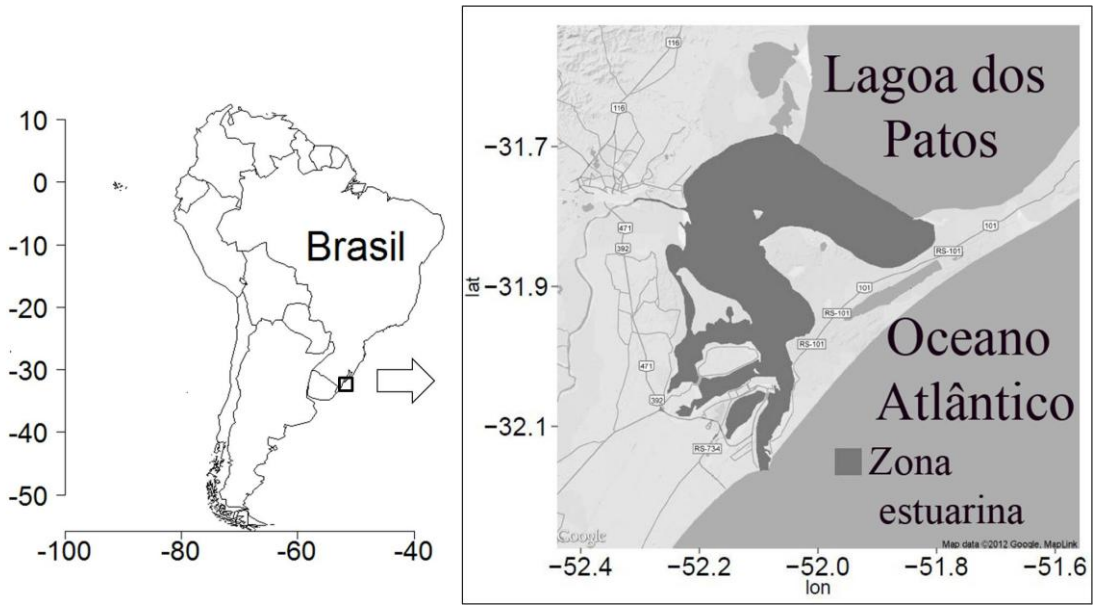

FIGURA 1 - Área de estudo. Lagoa dos Patos, RS, com destaque para a zona estuarina.

A região estuarina (figura 1) representa aproximadamente 10% da área da Lagoa e apresenta uma conexão com o Oceano Atlântico através de um estreito canal de 0,5 - 3,0 km de largura, 20 km de comprimento (Asmus 1997) e aproximadamente 12 m de profunidade (Möller et al. 2001). Em função da

proximidade de um ponto anfidrômico, o estuário apresenta um regime de micromarés (±0,4 m). Por este motivo, a entrada e a saída de água é controlada por vento e precipitação, com ventos do quadrante sul favorecendo a entrada de água salgada e ventos do quadrante norte e alta precipitação favorecendo o escoamento de água de origem continental (Hartmann & Schettini 1991, Möller et al. 2001, Soares & Möller Jr. 2001).

Os ventos do quadrante norte predominam na primavera e verão, enquanto que ventos do quadrante sul predominam no outono e inverno por causa da entrada de sistemas de frentes frias (Möller & Fernandes 2010). A descarga fluvial é baixa no verão e alta no final do inverno e início da primavera (Vaz et al. 2006). Esta sazonalidade de ventos e descarga causa uma sucessão no padrão de salinidade do estuário: cunha salina e parcialmente estratificado (primavera), verticalmente misturado (verão a outono), parcialmente estratificado (outono), altamente estratificado (inverno) e ausência de estuário (regime completamente fluvial devido à alta precipitação, inverno a primavera) (Hartmann & Schettini 1991).

Apesar deste padrão geral, o estuário é altamente dinâmico e a salinidade pode variar em curtos períodos (horas) em qualquer ponto (Hartmann & Schettini 1991, Möller & Fernandes 2010, Fujita & Odebrecht 2007, Abreu et al. 2010). O limite máximo de penetração da água salgada varia de acordo com regime de vento e descarga fluvial e em condições hidrológicas normais é até próximo à ponta da Feitoria (porção superior da área destacada na figura 1, aproximadamente a 75 km da entrada do estuário). Durante períodos prolongados de seca, a penetração da água salgada pode chegar a 150 km da

entrada do estuário ou, durante períodos de alta precipitação, a zona de mistura pode se transferir para a plataforma continental (Möller et al. 2001).

O estuário ainda situa-se em uma área sob forte influência do fenômeno ENSO, que promove alta precipitação (El Niño) ou seca (La Niña) (Grimm et al. 1998, Garcia et al. 2004, Abreu et al. 2010).

2.2 Amostragens e determinação dos parâmetros ambientais

Foram estabelecidas três tipos diferentes de amostragem: i) tipo-Lagrangiana (seguindo massas d'água de mesma faixa de salinidade), ii) Euleriana (ponto fixo) e transecto iii) (vários pontos ao longo do eixo longitudinal principal do estuário).

A amostragem tipo-Lagrangiana apresentada no primeiro trabalho (APÊNDICE 1) é uma adaptação da amostragem Lagrangiana, a qual consiste em seguir e coletar amostras em uma mesma parcela ou massa d'água em movimento (Hilmer & Imberger 2007). Esta estratégia é normalmente feita coletando-se amostras a bordo de uma embarcação ou boia à deriva (e.g. Trousselier et al. 2002). Diferente disto, amostras de água foram coletadas mensalmente, quando presentes, em três faixas de salinidade: limnética-oligohalina (0-1), mesohalina (14-16) e polihalina (28-31). Desta forma, ao longo do ano (junho de 2010 a maio de 2011) as coletas foram realizadas em diferentes pontos do estuário para se obter as salinidades apropriadas (pontos A-G, figura 2).

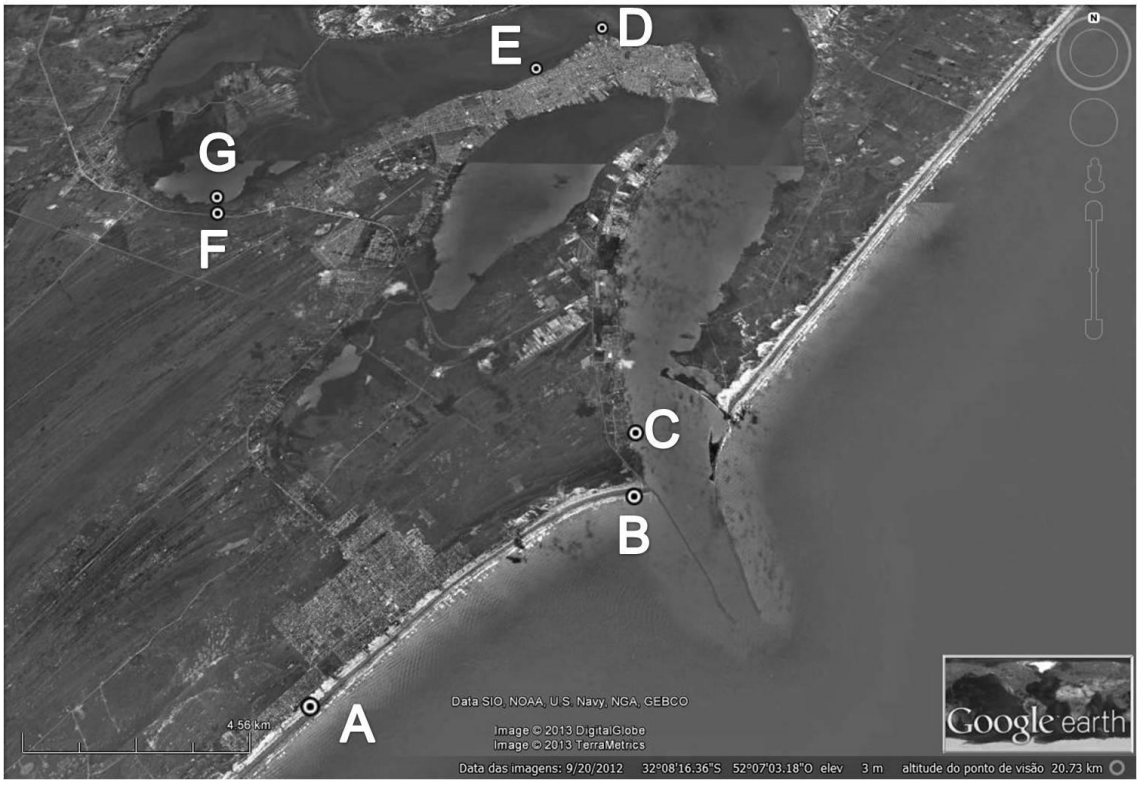

Figura 2 - Pontos amostrais da estratégia tipo-Lagrangiana (A-G) e Euleriana (C) no estuário da Lagoa dos Patos de junho de 2010 a maio de 2011. A - Praia do Cassino (Estação Marinha de Aquacultura); B - Praia do Cassino, (próximo ao molhe oeste); C - píer na Barra do Rio Grande; D - píer do Museu Oceanográfico; E - píer da Avenida Henrique Pancada; F - córrego do Saco do Justino; G - Saco do Justino. Fonte: Google Earth (2013).

No segundo trabalho (APÊNDICE 2) foi adotada uma estratégia de coleta Euleriana (ponto fixo) (e.g. Schult & Ducklow 2000). Amostras foram coletadas mensalmente na barra do Rio Grande de junho de 2010 a maio de 2011 (ponto C, figura 2).

No terceiro artigo foi empregada uma estratégia de coleta em transecto, sendo amostrados 9 pontos (A-I) ao longo do eixo longitudinal principal do estuário (figura 3). Amostras de água foram coletadas nos dias 23 e 24 de maio de 2012 a bordo da lancha Larus da Universidade Federal do Rio Grande

- FURG. No primeiro dia amostras superficiais foram coletadas do ponto A até o ponto H e no segundo dia foram coletadas (sentido I a B) amostras superficiais nas estações B, D, E, H, I e em profundidade (B, C, D, E, F, G).

Figura 3 - Pontos amostrais da estratégia de coleta por transecto no estuário da Lagos dos Patos nos dias 23 e 24 de maio de 2012. A - ponta dos molhes; B - Praticagem (Barra do Rio Grande); C - Avanti; D - Diamante; E - Setia; F - Barra de Pelotas; G - Nascimento; H - Porteiras; I - Ponta da Feitoria. Fonte: Google Earth (2013).

Em todos os três trabalhos foram coletadas amostras para determinar a composição e atividade da comunidade bacteriana e variáveis ambientais: salinidade, pH, temperatura, substâncias de baixo peso molecular, razão entre substâncias de alto e baixo peso molecular e os nutrientes inorgânicos NH_4^+,

NO_2^-, NO_3^- e PO_4^{-3}. No segundo e terceiro trabalhos foram determinados ainda o seston e a concentração de clorofila *a*.

As amostras superficiais foram coletadas com balde plástico e as amostras em profundidade do terceiro artigo foram coletadas cerca de 1 metro acima do fundo com garrafa de Niskin de 5 L de capacidade. No terceiro artigo os perfis verticais de temperatura foram determinados em cada ponto (Sonda YSI 30-50 FT). Nos demais trabalhos a temperatura (termômetro de mercúrio Incoterm) foi determinada na água imediatamente após a coleta. A salinidade foi medida para todos os pontos na água amostrada (balde ou garrafa) através de refractômetro (Atago S/Mill-E). Nas coletas em que foi determinado seston e clorofila *a*, uma parte da água (volumes variáveis) foi filtrada em filtros de fibra de vidro Whatman GF/F e os filtros foram imediatamente congelados a bordo (- 20 ºC); no segundo artigo a água foi filtrada para clorofila *a* em laboratório (< 3h após coleta). Uma parte da água foi pré-filtrada através de malha de plâncton de 5,0 µm de retenção para a exclusão da maioria dos metazoários, fitoplâncton e protozoários e acondicionada em frascos de borossilicato limpos e autoclavados (121 ºC por 30 minutos) em caixa térmica com gelo.

No laboratório foi determinado o pH (potenciômetro Digimed) em uma parte da água pré-filtrada. Outra alíquota da água pré-filtrada foi filtrada (250 mL) em membrana de policarbonato de 0,2 µm de retenção para a concentração de bactérias e a membrana foi congelada (- 20 ºC) imediatamente em microtubos Eppendorf estéreis.

Os frascos de coleta para bactérias e o aparato de filtração foram rinsados sequencialmente com as soluções: NaOH (0,1 M), EDTA (1 mM), solução fraca de hipoclorito de sódio, álcool etílico (70%), água destilada e água Milli-Q para

a remoção de ácidos nucléicos e inativação de nucleases. Três brancos foram feitos após este procedimento filtrando-se 250 mL de água destilada. Os filtros foram submetidos à extração de DNA e amplificação por PCR e nenhuma amplificação foi constatada. As pinças com que eram manipulados os filtros eram lavadas com estas soluções ou flambadas em chama.

A clorofila *a* foi extraída dos filtros com acetona 90% (v/v) a frio (-12 ºC) e medida por fluorometria (fluorímetro Turner TD700) sem acidificação (Welschmeyer 1994). O seston foi medido por gravimetria (Lenz 1972). Na água filtrada a concentração relativa de substâncias de alto e baixo peso molecular foi estimada através da absorbância a 365 e 250 nm, respectivamente, em espectrofotômetro UV-VIS Cary Varian (Strome & Miller 1978; Lindell et al. 1995; Stepanauskas et al. 2000). Os nutrientes NH_4^+ (Unesco 1983), NO_2^-, NO_3^- e PO_4^{-3} (Strickland & Parsons 1972) foram medidos por colorimetria.

2.3 Composição da comunidade bacteriana (CCB)

2.3.1 Princípio da Técnica de Eletroforese em gel com gradiente temporal de temperatura

A CCB foi analisada através do método de "fingerprinting" "Temporal Temperature Gradient Gel Electrophoresis" (TTGE) ou eletroforese em gel de poliacrilamida com gradiente desnaturante temporal de temperatura. Este método é uma variação do método "Denaturing Gradient Gel Electrophoresis" (DGGE), que se baseia na separação de fragmentos de DNA de tamanho similar através de eletroforese em gel de poliacrilamida desnaturante.

Diferentemente do DGGE, que emprega gradiente de desnaturação químico (uréia e formamida), o gel de TTGE possui uma concentração uniforme de uréia e o gradiente desnaturante é gerado pelo aquecimento do tampão de eletroforese a uma taxa constante ao longo da eletroforese. Os fragmentos vão sofrendo desnaturação à medida que migram no gel e apresentam redução de mobilidade ao atingirem a temperatura de “melting” (Tm), ou a temperatura em que metade das duplas hélices está dissociada. Esta temperatura é dependente da sequência de DNA (conteúdo de guaninas (G) e citosinas (C)) e por isso específica de cada espécie ou grupo de bactérias. As duplas fitas não se separam totalmente, pois é adicionada a um dos “primers” uma sequência de 40 GC, que possui alta Tm e não se dissocia nas temperaturas empregadas durante a eletroforese. Desta forma, o fragmento fica unido pelo grampo de GC. Esta conformação reduz muito a mobilidade do fragmento no gel e este pára em uma altura específica. Como resultado, forma-se no gel um conjunto de bandas que é correspondente a diferentes grupos taxonômicos de bactérias (unidades taxonômicas operacionais, UTO) (Muyzer 1999) (figura 4).

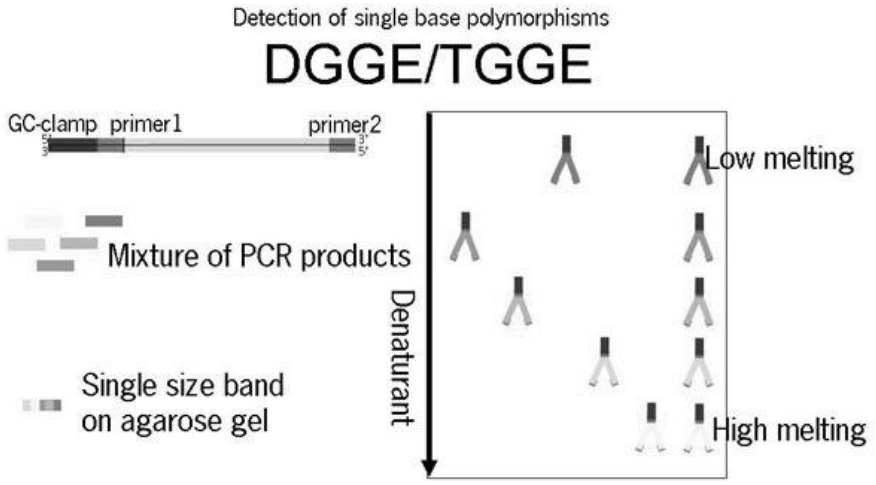

Figura 4 - Princípio do DGGE (“Denaturing Gradient Gel Electrophoresis”) e TGGE (“Temperature Gradient Gel Electrophoresis”). Disponível em: http://bib.convdocs.org/docs/1/790/conv_1/file1.pdf.

2.3.2 Extração de DNA

O DNA das bactérias concentradas nos filtros de policarbonato foi extraído com kit comercial QIamp DNA Stool (Qiagen) com a etapa de lise celular a 95 ºC. A qualidade e o tamanho do DNA foram checados através de eletroforese em gel de agarose 1%. O DNA extraído e purificado foi mantido congelado (-20 ºC) até análise.

2.3.3 Reação em cadeia da polimerase (“Polymerase Chain Reaction”, PCR)

A região hipervariável V3 dentro do gene que codifica a sub-unidade 16S do ribossomo bacteriano foi amplificada através de PCR em dois passos (“Nested-PCR”): no primeiro foram utilizados os “primers” 11F (5’-GTTTGATCCTGGCTCAG-3’) e 1492R (5’-TACCTTGTTACGACTT-3’) que amplificam quase todo o gene 16S (Siripong & Rittman 2007) e no segundo

passo foram empregados os “primers” GC-338F (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG - 3') e 518R (5'- ATT ACC GCG GCT GCT GG - 3'), que geram fragmentos de aproximadamente 180 pares de base (Henriques et al. 2006).

No primeiro passo da PCR as condições foram: concentração final: 1× Tampão de reação (inicial 10×: 20 mM Tris-HCl (pH 8,0), 40 mM NaCl, 2 mM fosfato de sódio, 0,1 mM EDTA, 50 % v/v glicerol), 1,5 mM $MgCl_2$, 0,2 mM de cada dNTP, 0,2 µM de cada “primer”, 20 ng μL^{-1} de BSA, 0,1 µL de Platinum® Taq DNA polimerase (5 U μL^{-1}, Invitrogen) e 1,0 µL de DNA molde em 12,5 µL de volume de reação. As condições da amplificação foram: 5 minutos a 94°C; 30 ciclos de 5 minutos a 94 °C, 1 minuto a 52 °C e 1 minuto e 30 s a 72 °C; extensão final de 10 minutos a 72 °C.

O segundo passo foi feito nas seguintes condições: concentração final: 1× tampão de amplificação (inicial 10×: 20 mM Tris-HCl (pH 8,0), 12 mM $MgSO_4$, 40 mM KCl, 1 mM DTT, 0,1 mM EDTA, 50% v/v glicerol), 0,3 mM de cada dNTP, 0,3 µM de cada iniciador, 1,0 µL de Pfx 50™ DNA polimerase (5 U μL^{-1}, Invitrogen) e 2,0 µL de DNA molde em um volume total de 50 µL. A amplificação foi feita nas seguintes condições: 5 minutos a 94 °C; 20 ciclos decrescentes (van der Gucht et al. 2007) com 1 minuto a 94 °C, 1 minuto começando em 65 °C e terminando em 55 °C (decrescendo 0,5 °C cada ciclo) e 1 minuto a 68 °C; 20 ciclos: 1 minuto a 94 °C, 1 minuto a 55 °C, 1 minuto a 68 °C; 30 minutos a 68 °C de extensão final de forma a minimizar a formação artefatual de bandas (Janse et al. 2004). Os produtos de PCR foram checados quanto ao tamanho esperado através de eletroforese em gel de agarose 1% e

posteriormente quantificados com o kit Quant-It™ dsDNA broad range assay kit (Invitrogen).

2.3.4 Protocolo empregado para a análise de TTGE

A análise TTGE foi realizada com o sistema de eletroforese DCode Universal Mutation Detection System (BioRad) (figura 5), baseado nas recomendações do fabricante e várias modificações empiricamente testadas: gel de poliacrilmida 14% (37,5:1 acrilamida:bis-acrilamida), uréia 7M e tampão TAE 1.25×. Os géis foram polimerizados com a adição de 50 µL de TEMED e 100 µL de APS 10% por 1,5 a 2 h e submetidos a uma pré-corrida a 200 V por aproximadamente 30 minutos. As amostras (~ 600 ng de produto de PCR) foram aplicadas na proporção de 1:1 amostra:corante de carga (70% glicerol, 0,05% azul de bromofenol, 0,05% xilenocianol). As condições de corrida foram: de 66,0 a 69,7 °C, com incrementos 0,2 °C h^{-1} a 68 V (Lehours *et al.* 2010). Os géis foram corados em tampão TAE 1.25× contendo 1 µg $µL^{-1}$ de brometo de etídio por 15 minutos e descorados em tampão TAE 1.25× pelo mesmo tempo. Os géis foram fotografados em transiluminador acoplado a sistema de captura de imagem (T1201 Sigma/Ultra Lum Ultra Cam Digital Imaging equipment acoplado à câmera Power Shot A620 Canon) com excitação ultravioleta. A calibração e o alinhamento entre géis foram feitos com o auxílio de uma amostra padrão contendo seis isolados bacterianos (equivalente ao “clone ladder” descrito em Tourlomousis *et al.* 2010) previamente obtidos do estuário. Para a confecção deste padrão, foram misturados aproximadamente 100 ng de DNA amplificado de cada isolado e a mistura aplicada antes e após as amostras da mesma maneira que as mesmas.

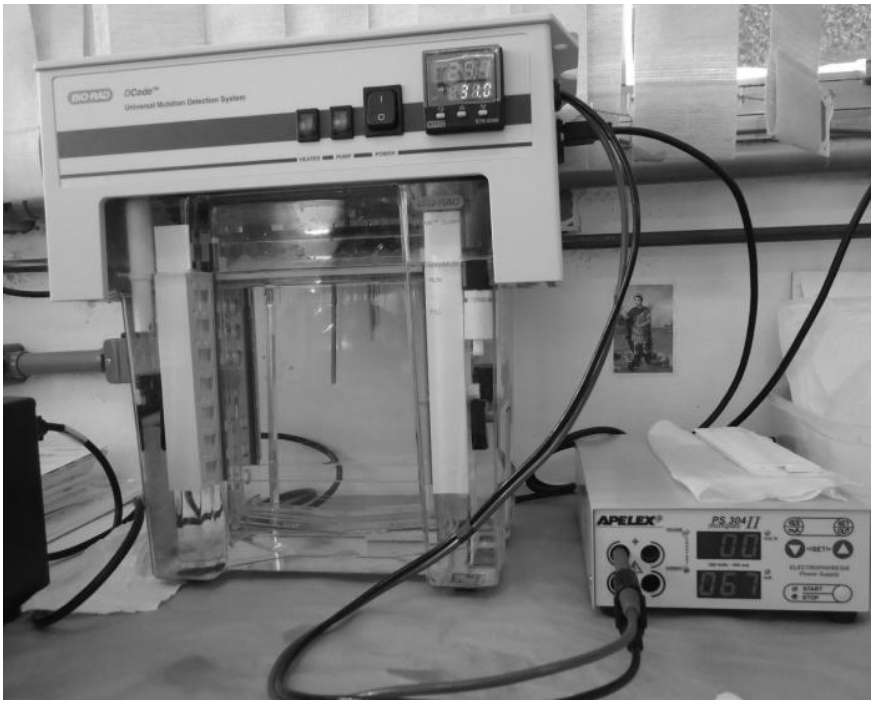

Figura 5 - Equipamento DCode BioRad para eletroforese em gel de poliacrilamida com gradiente desnaturante temporal de temperatura.

2.3.5 Análise de Imagens

A detecção de bandas e a correção das alturas das bandas nas imagens foi feita com o auxílio do programa Gel Analyzer 2010a (Lazar, 2010) após subtração de fundo do tipo “rolling ball”. As bandas foram detectadas automaticamente e posteriormente corrigidas por supervisão. A posição das bandas foi corrigida através de valores de referência (Vr, uma medida da altura da banda relativa ao comprimento total da coluna ao longo da qual os fragmentos migram) utilizando o padrão de isolados. Os valores de referência são linhas suaves que ligam pontos com mesmo Vr ao longo de vários géis. Como resultado, o programa Gel Analyzer fornece uma matriz com a posição relativa e o volume bruto (altura do pico × área do pico de fluorescência) de

cada banda. O volume bruto é utilizado apenas para o alinhamento entre as bandas (seção 2.4.6) entre colunas diferentes e é gerado automaticamente pelo programa.

2.3.6 Alinhamento e construção das matrizes de presença/ausência

Uma mesma posição de banda pode apresentar pequenas variações em diferentes amostras, por isso é necessário realizar um alinhamento que leva em consideração um nível de tolerância dentro do qual bandas em posições ligeiramente diferentes em diferentes colunas são considerados como o mesmo UTO. Este procedimento foi feito utilizando-se os valores de Vr e volume bruto (volume integrado de intensidade de banda) através de um algoritmo de alinhamento de picos baseado em método de agrupamento (Ishii et al. 2009) no programa R 2.15.0 (R Development Core Team, 2012). O nível de tolerância foi estipulado em 1% (“cutoff” = 0.01). Os valores brutos foram convertidos em presença/ausência.

2.4 Perfis fisiológicos da comunidade bacteriana (PFCB)

2.4.1 Princípio do método - ensaio de capacidade de consumo de substratos (Ecoplate™)

Este ensaio foi feito nas microplacas comerciais Ecoplate™ (Biolog), que possuem 31 substratos orgânicos diferentes (tabela 1) e junto com os substratos o corante indicador redox tretrazólio violeta. Um poço adicional funciona como um branco e possui apenas o corante indicador redox. Quando ocorre oxidação do substrato, há formação de compostos doadores de elétrons

(NADH, por exemplo) que reduzem o corante e este adquire uma coloração púrpura que pode ser quantificada colorimetricamente, indicando capacidade e intensidade de uso do substrato (figura 6). Estes substratos estão dispostos em triplicatas (3 conjuntos de 31 substratos + poço controle).

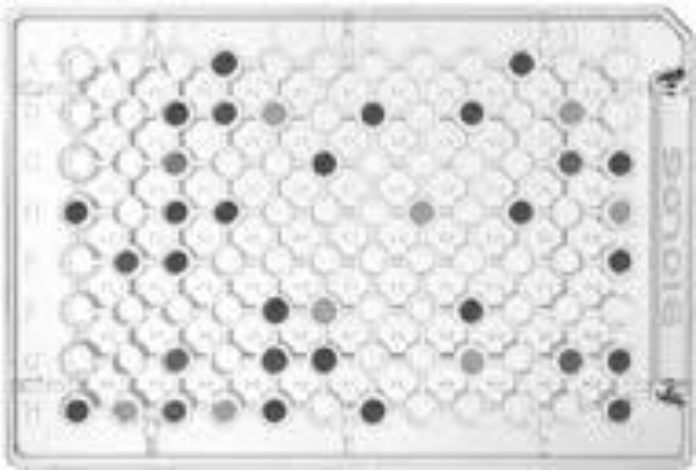

Figura 6 - Esquema de uma placa Ecoplate™ inoculada onde podem ser vistos os poços em que houve oxidação dos substratos (poços escuros), com diferentes intensidades. Disponível em: .http://sites.google.com/site/cellbiosciencesau/services/biolog-1.

Tabela1 - Lista de substratos orgânicos encontrados nas Ecoplates™ (Biolog) com características químicas gerais. PM, peso molecular; FN, fonte nutricional (C - carbono, N - nitrogênio, P - fósforo)

Substrato	Grupo químico[1]	PM[2]	FN[3]
putrescina	amina	88,15	C+N
ácido α-cetobutírico	ácido carboxílico	102,09	C
metil piruvato	éster	102,09	C
ácido γ-hidroxibutírico	ácido carboxílico	104,10	C
L-serina	aminoácido	105,09	C+N
L-treonina	aminoácido	119,12	C+N
feniletilamina	amina	121,18	C+N
i-ertritol	carboidrato	122,12	C
ácido itacônico	ácido carboxílico	130,10	C
L-asparagina	aminoácido	132,12	C+N
ácido D-málico	ácido carboxílico	134,09	C
ácido 2-hidroxibenzoico	ácido carboxílico	138,12	C
ácido 4-hidroxibenzoico	ácido carboxílico	138,12	C
D-xilose	carboidrato	150,13	C
L-fenilalanina	aminoácido	165,19	C+N
D,L-α-glicerol fosfato	químico fosforilado	172,07	C+P
L-arginine	aminoácido	174,20	C+N
ácido D-galactônico- γ lactona	ácido carboxílico	178,14	C
α-D-galactose	carboidrato	180,16	C
D-manitol	carboidrato	182,17	C
ácido D-galacturônico	ácido carboxílico	194,14	C
β-metil-D-glicosídeo	carboidrato	194,18	C
ácido D-glicosamínico	ácido carboxílico	195,17	C+N
ácido glicil-L-glutâmico	aminoácido	204,18	C+N
N-acetill-D-glicosamina	carboidrato	221,21	C+N
Glicose-1-fosfato	químico fosforilado	260,14	C+P
D-Celobiose	carboidrato	342,30	C
Tween 80	polímero	604,81	C
Tween 40	polímero	620,86	C
Glicogênio	polímero	666,58	C
α-Ciclodextrina	polímero	972,84	C

1 - Garland & Mills (1991).

2 - National Center for Biotechnology Information. PubChem Compound Database. Disponível em: <http://pubchem.ncbi.nlm.nih.gov>.

3 - Sala et al. (2006).

2.4.2 Incubação

Todo o procedimento foi realizado em câmara de fluxo lâminar, utilizando ponteiras com barreira estéreis para evitar contaminação. A água pré-filtrada foi inoculada (150 µL) em cada poço e as placas foram incubadas a 20 ºC por sete dias no escuro.

2.4.3 Leitura

Ao final da incubação, os poços foram individualmente homogeneizados com micropipeta automática e as placas foram lidas a 595 nm em leitor de microplacas (TP Reader NM Thermoplate).

2.4.4 Padronização dos dados

As leituras finais foram subtraídas da leitura do poço controle, sendo considerado como zero os valores negativos. Estas leituras corrigidas foram divididas pelo AWCD (“Average Well Colour Development”), que é a média aritmética de todas as leituras da placa após a correção dos zeros. Este procedimento reduz o efeito de densidades iniciais diferentes do inóculo (Garland & Mills 1991).

2.4.4.1 Índices Nuse e Puse

Dois índices foram calculados com base no padrão de consumo de substratos: Nuse e Puse. O índice Nuse é calculado como a soma dos valores de absorbância dos compostos à base de nitrogênio (N) em relação à soma total de todos os substratos após a correção dos zeros (sem padronização AWCD). Este índice foi testado experimentalmente e é um indicador de

limitação por nitrogênio, aumentando nestas condições; em amostras ambientais, está negativamente correlacionado à concentração de nitrogênio inorgânico dissolvido (Sala et al. 2006).

De forma similar, no presente trabalho foi proposto o índice Puse, calculado da mesma forma que o Nuse, mas levando-se em conta substratos à base de fósforo (P). A lista de substratos com a base da fonte nutricional (N ou P) encontra-se na tabela 1.

2.5 Análises estatísticas

No primeiro artigo, a similaridade da composição da comunidade bacteriana e do padrão de consumo de substratos entre as amostras das diferentes faixas de salinidade foi analisada através de análise de agrupamento (algoritmo “Unweighted Pair Group Method With Arithmetic Mean”) no programa R 2.15.0 (R Development Core Team 2012). Para: i) a composição da comunidade foi empregada a medida de dissimilaridade Bray-Curtis nos dados de presença/ausência após exclusão de bandas com frequência de ocorrência < 10%; Para ii) o padrão de consumo de substratos, foi calculada a dissimilaridade da correlação (1- correlação de Pearson) baseados nos valores de AWCD (Hackett & Griffiths 1997). Diferenças estatísticas entre as faixas de salinidades foram testadas através de Análise de Similaridade (ANOSIM) com p-valores estimados por teste de permutação (1.000 permutações) no programa PAST 2.14 (Hammer *et al*. 2001). Análises de Correspondência Canônica (CCA) foram realizadas para identificar as variáveis ambientais (temperatura, pH, NH_4^+, NO_2^- , NO_3^-, PO_4^{-3}, substâncias de baixo peso

molecular e razão entre substâncias de baixo e alto peso molecular) com efeito significativo sobre a composição da comunidade e a atividade bacterianas. A salinidade foi excluída da CCA pelo direcionamento de valores específicos desta variável em função do tipo de coleta empregado. Da mesma forma que para a análise de agrupamento, as bandas com frequência < 10% foram excluídas. A seleção de variáveis foi feita pela análise dos fatores de inflação ("variance inflation factors", VIFs), a significância global da CCA e dos eixos canônicos individualmente foi testada por permutação (α = 0,05, 1.000 permutações). Este procedimento foi baseado em Borcard *et al.* (2011) usando o programa R 2.15.0 (R Development Core Team, 2012) e os pacotes Vegan (Oksanen et al. 2011) e BiodiversityR (Kindt & Coe 2005).

No segundo artigo, a variação temporal da composição e atividade da comunidade bacterianas e a influência de fatores ambientais (salinidade, temperatura, pH, profundidade de Secchi, NH_4^+, NO_2^- , NO_3^-, PO_4^{-3} e substâncias de baixo peso molecular) sobre a composição e a atividade bacterianas foram investigadas através de análise de agrupamento e CCA, respectivamente, utilizando os mesmos critérios descritos para o primeiro artigo. A diferença estatística entre grupos de amostras do primeiro (P1: Junho a Outubro de 2010, sob influência de El Niño) e o segundo período amostral (P2: Novembro de 2010 a Maio de 2011, sob influência de La Niña) foram testadas através de Análise de Variância Multivariada Não Paramétrica (NPMANOVA) (Anderson 2001) utilizando os mesmos índices de similaridade/dissimilaridade empregados nas análises de agrupamento através do programa PAST 2.14 (Hammer *et al.* 2001), sendo o valor de p obtido através de 9.999 permutações e corrigido pelo método de Bonferroni (n × p,

sendo n o número de comparações e p o valor de probabilidade obtido por permutação). Diferenças entre P1 e P2 para as variáveis ambientais (salinidade, temperatura, pH, profundidade de Secchi, clorofila *a*, NH_4^+, NO_2^- , NO_3^-, PO_4^{-3} e substâncias de baixo peso molecular e índices Nuse e Puse) foram testadas através de teste t com valor de p obtido por 10.000 permutações através do pacote Permute (Simpson 2012) e a função pt.test (codificada em: http://127.0.0.1:26109/library/permute/html/shuffle.html) no programa R 2.15.0 (R Development Core Team 2012). A correlação cruzada entre a salinidade e o índice SOI (ìndice Oscilação Sul, que indica eventos de El Niño e La Niña) foi testada através de correlação cruzada no programa PAST 2.14 (Hammer *et al.*, 2001).

No terceiro artigo, a variação espacial e temporal da composição e atividade bacterianas e o efeito de fatores ambientais (temperatura, pH, profundidade de Secchi, seston, clorofila *a*, substâncias de baixo peso molecular, NH_4^+, NO_2^- , NO_3^-, PO_4^{-3}) sobre a composição e atividade bacterianas foram investigadas através de análise de agrupamento e análise de correspondência canônica, respectivamente, utilizando os mesmos critérios descritos para o primeiro artigo e utilizando o programa R 3.0.0 (R Development Core Team 2013). A diferença estatística entre amostras agrupadas por dia ou profundidade foram testadas por NPMANOVA da mesma forma que descrito para o segundo artigo. Para os índices Nuse e Puse foi utilizada a distância euclidiana como medida de dissimilaridade.

3. SÍNTESE DOS RESULTADOS

3.1 "Stability of bacterial composition and activity in different salinity waters in the dynamic Patos Lagoon estuary: Evidence from a Lagrangian-like approach"

Ng Haig They, Lise Maria Holanda Ferreira, Luís Fernando Marins e Paulo Cesar Abreu.

Trabalho aceito para publicação na revista Microbial Ecology (16/05/2013)

A composição da comunidade bacteriana (CCB, TTGE) e os perfis fisiológicos da comunidade bacteriana (PFCB, Ecoplate™) foram estudados através de uma amostragem tipo-Lagrangiana em três faixas de salinidade (limética-oligohalina, 0-1; mesohalina, 14-16 e polihalina, 28-31), no estuário da Lagoa dos Patos e zona costeira mensalmente entre junho de 2010 e maio de 2011. Em cada evento amostral a salinidade foi medida em vários pontos e as amostras foram coletadas sempre que a salinidade da amostra esteve dentro dos intervalos considerados. Uma série de variáveis ambientais também foi medida: pH, temperatura, substâncias de baixo peso molecular (SBPM), razão entre substâncias de alto e baixo peso molecular (SAPM:SBPM) e os nutrientes inorgânicos NH_4^+, NO_2^- e NO_3^-. Os dados foram analisados através de análises de agrupamento e a influência das variáveis ambientais sobre CCB e PFCB foi avaliada através de análise de correspondência canônica (ACC). Um total de 24 amostras foi coletado, sendo 9 na faixa limnética-oligohalina, 5 na faixa mesohalina e 10 na faixa polihalina. A análise de agrupamento revelou grupos específicos de amostras por faixa de salinidade para CCB, mesmo para

amostras coletadas em locais e meses diferentes. Para PFCB, houve agrupamento das amostras mesohalinas e polihalinas, que por sua vez foram distintas das amostras limnético-oligohalinas. Da mesma forma estes agrupamentos contiveram amostras coletadas em locais e meses diferentes. O PFCB do grupo mesohalino-polihalino apresentou um número menor de substratos oxidados, mas alguns foram metabolizados em maior intensidade; a maior riqueza de substratos oxidados nas amostras oligohalinas sugeriu que a atividade das bactérias foi maior em água mais doce, decrescendo em água mais salina. Dentre os substratos utilizados em maior intensidade pelo grupo mesohalino-polihalino, destacaram-se os carboidratos e aminoácidos; este resultado, associado ao maior índice Nuse (baseado no consumo relativo de compostos nitrogenados em relação ao total) e a importância de NO_2^- e NO_3^-, SBPM e SAPM:SBPM indicou uma possível limitação por nutrientes e substratos nas águas de maior salinidade. Em geral houve: i) alta estabilidade da CCB e PFCB, um resultado inesperado para um ambiente dinâmico como a Lagoa dos Patos; ii) diferenças significativas entre CCB de águas das três faixas de salinidades, em concordância com dados de literatura; iii) diferenças significativas dos PFCB entre águas mais salinas (meso- a polihalinas) e menos salinas (liméticas-oligohalinas), indicando diferenças de atividade entre as mesmas. Conclui-se que processos ecológicos microbianos distintos ocorrem em águas com diferentes salinidades no estuário da Lagoa dos Patos.

(*O trabalho completo em Inglês encontra-se no Apêndice 1*)

3.2: "Bacterioplankton phylogenetic and physiological shifts associated to the El Niño Southern Oscillation (ENSO) in the Patos Lagoon estuary"

Ng Haig They, Lise Maria Holanda Ferreira, Luís Fernando Marins e Paulo Cesar Abreu.

Trabalho submetido à revista FEMS Microbiology Ecology (06/05/2013)

A composição da comunidade bacteriana (CCB, TTGE) e os perfis fisiológicos da comunidade bacteriana (PFCB, Ecoplate™) foram investigados durante uma transição entre um evento moderado de El Niño para um evento forte de La Niña no estuário da Lagoa dos Patos. Amostras mensais foram coletadas entre em um ponto fixo (amostragem Euleriana) próximo à saída do estuário mensalmente entre junho de 2010 a maio de 2011. Variáveis ambientais foram também medidas: pH, salinidade, temperatura, transparência (profundidade de Secchi), clorofila *a* e substâncias de baixo peso molecular (SBPM). A variação temporal da CCB e da PFCB foi investigada através de análise de agrupamento e o efeito das variáveis ambientais sobre a CCB e a PFCB foi testado através de análise de correspondência canônica (ACC). A salinidade foi correlacionada com o Índice Oscilação Sul (IOS) com um lapso temporal de 4 meses, apresentando dois períodos marcados: um de baixa salinidade (0-15) que se seguiu após o término de um evento de El Niño (Período 1, P1: de junho a outubro de 2010) e outro de alta salinidade (17-36) durante e após um evento de La Niña (Período 2, P2: de novembro de 2010 a maio de 2011). Em resposta a essas mudanças nas condições ambientais, a CCB e a PFCB apresentaram diferenças marcantes e significativas entre P1 e P2. Houve um aumento de temperatura, transparência e clorofila e diminuição das

concentrações de NO_3^- e NO_2^-, PO_4^{-3} e SBPM no P2 em relação ao P1. O PO_4^{-3}, porém apresentou tendência a aumentar novamente após janeiro de 2011, enquanto o teor de NH_4^+ aumentou gradativamente após novembro de 2010, atingindo um pico em março de 2011. Estes dois resultados, associados a maiores valores dos índices Nuse e Puse (índices baseados no consumo relativo de substratos nitrogenados e fosfatados, respectivamente, em relação ao consumo total dos substratos) em situações de menores valores de nutrientes, sugeriram um maior efeito de decomposição/mineralização durante a fase La Niña. Outra possibilidade para explicar o acúmulo de íon amônio seria falhas na nitrificação, uma vez que houve lapsos temporais entre o aumento da concentração de NH4+ e a concentração de NO_2^-. Os resultados mostraram que a CCB e a PFCB respondem a hidrologia e variáveis ambientais reconhecidamente influenciadas pelo fenômeno El Niño, o que sugere uma ligação entre a dinâmica de bactérias no estuário da Lagoa dos Patos e este fenômeno climático de escala global.

(*O trabalho completo em Inglês encontra-se no Apêndice 2*)

3.3: “Distinct responses of bacterioplankton community composition and activity to salinity and nutrients gradients along the microtidal Patos Lagoon estuary”

Ng Haig They, Lise Maria Holanda Ferreira, Luís Fernando Marins, Osmar Olinto Möller Jr. e Paulo Cesar Abreu.

Trabalho em preparação para ser submetido à Revista Estuarine Coastal and Shelf Science

A variação espacial ao longo do eixo longitudinal principal (amostragem em transecto: ~74 km) e temporal de curta escala (23 e 24/05/2012) foi avaliada no estuário da Lagoa dos Patos. Foram avaliadas a composição da comunidade bacteriana (CCB, TTGE) e os seus perfis fisiológicos (PFCB, Ecoplate™) em 8 estações coletadas na superfície no primeiro dia e 5 estações coletadas na superfície e 6 estações coletadas em profundidade (1 metro acima do sedimento) no segundo dia. Das amostras do segundo dia, em 3 estações foram coletadas amostras simultaneamente em superfície e fundo. Variáveis ambientais foram também medidas: pH, temperatura, salinidade, transparência (disco de Secchi), clorofila *a*, seston, substâncias de baixo peso molecular (SBPM) e nutrientes (NH_4^+, NO_2^-, NO_3^- e PO_4^{-3}). O padrão espacial e temporal da CCB e da PFCB foi investigado através de análise de agrupamento e a influência das variáveis ambientais sobre CCB e PFCB foi testada através de análises de correspondência canônica (ACC). Os resultados demonstraram uma variação espacial da CCB e da PFCB, em função do gradiente de salinidade. Houve diferenças significativas entre os dias e entre amostras de superfície e fundo. As condições do primeiro dia foram mesohalinas, com

baixas concentrações de nutrientes e seston; no segundo dia, a entrada de cunha salina trouxe condições polihalinas, elevando as concentrações de nutrientes (notadamente NO_2^-. NO_3^- e PO_4^{-3}) e principalmente seston e clorofila *a* nas estações mais próximas da entrada do estuário e em profundidade, o que indicou ressuspensão. Houve também aumento significativo dos índices Nuse e Puse (índices baseados no consumo relativo de substratos nitrogenados e fosfatados, respectivamente, em relação ao consumo total dos substratos) no segundo dia. Outras explicações incluem o deslocamento de água instersticial rica em nutrientes pela água salgada mais densa e o retorno de água estuarina previamente exportada. A variação da CCB provavelmente se deu pela combinação de crescimento em resposta a mudanças na salinidade, nutrientes e substratos, bem como pela entrada de bactérias costeiras e ressuspendidas. A PFCB, por sua vez, também respondeu a variações de salinidade, nutrientes e substratos e possivelmente foi afetada pelo metabolismo diferenciado de bactérias aderidas ressuspensas. O aumento dos índices Nuse e Puse em situação de maior quantidade de nutrientes sugeriu que o metabolismo bacteriano pode ter apresentado um lapso temporal na resposta ao aumento da concentração de nutrientes ou pode ter havido uma maior contribuição do metabolismo de bactérias aderidas mais ativas. Interessantemente, a PFCB indicou maior atividade em águas mais salinas, contrariando estudos prévios. A ocorrência de maior quantidade de nutrientes em águas com maior salinidade sugeriu que a atividade pode ser uma resposta primária a nutrientes e substratos. Desta forma, a CCB e a PFCB parecem responder a diferentes conjuntos de variáveis ambientais; a CCB primariamente à salinidade e a PFCB primariamente à disponibilidade de nutrientes e substratos.

(*O trabalho completo em Inglês encontra-se no Apêndice 3*)

4. DISCUSSÃO GERAL

A conexão entre os três trabalhos desenvolvidos na presente tese está no fato de que os três estudos fizeram a mesma avaliação da composição e atividade das comunidades bacterianas analisadas, mas através de três tipos diferentes de amostragem. O emprego de diferentes estratégias amostrais visou determinar o efeito das diferentes escalas espaciais e temporais, bem como o papel das variáveis ambientais (salinidade, nutrientes e substratos) sobre a composição (CCB) e os perfis fisiológicos da comunidade bacteriana (PFCB) no estuário da Lagoa dos Patos e região costeira. As variáveis ambientais, por sua vez, são determinadas pela hidrodinâmica, o que sugere uma ligação forte, mas provavelmente secundária, desta com a dinâmica da diversidade e atividade bacterianas.

4.1Tipo de amostragem

No primeiro trabalho desenvolvido (APÊNDICE 1), foi empregada uma amostragem tipo-Lagrangiana para responder à pergunta: existe estabilidade na CCB e PFCB em três faixas de salinidade? A coleta de amostras utilizando faixas de salinidade como unidades amostrais permitiu investigar o grau de similaridade da composição da comunidade bacteriana (CCB) e perfis fisiológicos da comunidade bacteriana (PFCB) em amostras de água coletadas em locais e meses diferentes, mas sempre na mesma faixa de salinidade.

Os trabalhos com bactérias em estuários utilizando estratégia Lagrangiana são escassos. Alguns poucos exemplos incluem modelagem matemática

(Salomon & Pommepuy 1990; Pierini et al. 2012) de taxa de depuração de indicadores biológicos de qualidade da água como a bactéria *E. coli*, por exemplo. Destaca-se, no entanto, um trabalho que avaliou a diversidade e atividade bacterianas através de uma estratégia Lagrangiana típica utilizando um flutuador na pluma estuarina do rio Ródano, França. Neste trabalho, Trousselier et al. (2002) investigaram mudanças na composição da comunidade bacteriana ("Denaturing Gradient Gel Electrophoresis", DGGE) e atividade (produção secundária e DGGE a partir de RNA) ao longo do gradiente salino com variações na salinidade (0-38) e, conforme esperado, foram encontradas variações significativas na composição das comunidades, enquanto que a atividade bacteriana decresceu na zona de mistura, aumentando em direção aos extremos de salinidade. A estratégia puramente Lagrangiana permite, portanto, a avaliação da estabilidade da composição e atividade da comunidade bacteriana em uma mesma massa ou parcela d'água, mas engloba mudanças decorrentes da mistura de água doce e água salgada que ocorrem ao longo do gradiente salino.

A abordagem tipo-Lagrangiana apresenta, no entanto, algumas limitações. Primeiramente, seria ideal o conhecimento da variação da salinidade em escalas temporais e espaciais finas, de modo a determinar as variações prévias de salinidade que uma determinada parcela de água a ser amostrada já sofreu. Isto é particularmente crítico para a faixa mesohalina, pois ela pode resultar de processos de mistura que ocorrem em diferentes escalas temporais. Esta limitação não ocorre para as faixas limnético-oligohalina e polihalina, onde a mistura de águas com salinidades diferentes é mínima, ou nula.

A interpretação dos resultados no caso de mistura ou ausência de mistura é diferente. Quando não há mistura, a manutenção de uma composição de comunidade ou atividade ao longo do tempo indica apenas estabilidade, enquanto que na presença de mistura e alta variabilidade da salinidade, além da estabilidade haverá também resiliência, pois a comunidade bacteriana terá sido submetida a perturbações de salinidade, mas retorna à composição original, ou próxima a estas mesmas condições (Begon et al. 2006).

No segundo trabalho (APÊNDICE 2), a variabilidade temporal de média escala (sazonal) da salinidade foi determinante para os padrões encontrados para a CCB e PFCB e, portanto, a amostragem Euleriana mostrou-se a mais adequada para a questão colocada, qual seja: a composição das comunidades de bactérias e sua atividade respondem a diferentes escalas temporais e espaciais, inclusive a fenômenos climáticos globais que afetam a hidrodinâmica do estuário, tais como o ENSO?

Coletas de água em um único ponto ao longo do tempo foi a estratégia utilizada para medidas de diferentes parâmetros da comunidade bacteriana, como densidade e biomassa de bactérias livres e aderidas no estuário da Lagoa dos Patos (Abreu et al. 1992), bem como a determinação da produção secundária de bactérias livres e aderidas no estuário do rio Sena, França (Servais & Garnier 2006). Ainda foi utilizada para estudar a atividade bacteriana através da capacidade de degradação de compostos orgânicos (Ecoplate™) no rio York, EUA (Schultz & Ducklow 2000). Em Abreu et al. (1992) e Schultz & Ducklow (2000) importantes variações sazonais (influência de meses do ano e temperatura) foram evidentes indicando ser esta estratégia amostral mais

indicada para a caracterização de variabilidade temporal de média- e larga-escala.

No terceiro trabalho (APÊNDICE 3), a variabilidade espacial (longitudinal) e temporal de curta escala foi avaliada através da coleta de amostras de água em vários pontos dispostos ao longo de um gradiente halino, o que revelou diferenças na CCB e PFCB entre estações e entre a superfície e o fundo de algumas estações (variação espacial) e entre um dia e outro de coleta (variação temporal). Esta abordagem permitiu a caracterização quase simultânea de diferentes condições ambientais que ocorrem ao longo de gradientes salinos e é, por isto, a mais comumente utilizada em trabalhos de ecologia microbiana em estuários (e.g. Crump et al. 2004, Bouvier & del Giorgio 2002, del Giorgio & Bouvier 2002, Kirchman et al. 2005, Herlemann et al. 2011, Campbell & Kirchman 2012, Fortunato et al. 2012). Estes estudos têm demonstrado padrões de variação espacial da diversidade bacteriana (biogeografia) (Crump et al. 2004, Kirchman et al. 2005, Herlemann et al. 2011), atividade (del Giorgio & Bouvier 2002, Campbell & Kirchman 2012) e variações sazonais (Fortunato et al. 2012).

Desta forma, a utilização de três estratégias distintas de amostragem se mostrou eficaz no sentido de responder às perguntas propostas e a avaliação de novas metodologias que foram aplicadas em diferentes escalas de espaço e tempo. Entretanto, nos três trabalhos ficou clara a ação hidrodinâmica e da salinidade na estruturação das comunidades bacterianas e de sua atividade.

4.2 Efeito da hidrodinâmica sobre condições ambientais

Os resultados do segundo (APÊNDICE 2) e terceiro trabalhos (APÊNDICE 3) claramente indicaram a influência da hidrodinâmica na diversidade e atividade das bactérias no estuário da Lagoa dos Patos.

No segundo trabalho, a transição de condições El Niño moderado para La Nina forte foi associada a condições distintas de salinidade, nutrientes e substratos na Lagoa dos Patos. O efeito do fenômeno de escala global El Niño Oscilação Sul (El Niño Southern Oscillation – ENSO) sobre a hidrologia do estuário da Lagoa dos Patos é conhecido. Sabe-se que situações de aquecimento de águas do Pacífico Oriental (El Niño) causam aumento de precipitação em toda a bacia de drenagem da Lagoa dos Patos, enquanto que condições de menor chuva ocorrem durante períodos de resfriamento das águas do Pacífico (La Niña), sendo que este efeito se estende por todo o sul do Brasil (Grimm et al. 1998).

Como resultado deste fenômeno de larga escala, tem sido observada prolongada salinização do estuário associada à fase La Niña (Garcia et al. 2004; Abreu et al. 2010). Este fenômeno tem sido relacionado a alterações na biomassa e composição da comunidade do fitoplâncton (Abreu et al. 2010), distribuição e composição de assembleias de peixes (Garcia et al. 2001), distribuição do mexilhão dourado *Limnoperna fortunei* (Capítoli et al. 2008), recrutamento da grama marinha *Ruppia maritima* (Odebrecht et al. 2010), bem como abundância de espécies do macrozoobentos (Colling et al. 2007, Odebrecht et al. 2010), pesca (Garcia et al. 2004) e captura de camarões (Castello & Möller 1978, Möller et al. 2009).

Para as bactérias, uma relação positiva entre a densidade e precipitação foi apontada por Cesar (2002), sugerindo como mecanismo principal a diminuição da predação por diluição dos predadores, mas também ao maior aporte de nutrientes. Este padrão foi observado em um estudo de cinco anos que englobou um forte evento de El Niño em 1997 e 1998. Não há registro, porém, de trabalhos que associem o fenômeno ENSO à diversidade e atividade de bactérias estuarinas (conforme busca na base Web of Knowledge: “bacteria” (topic) + “estuary” (topic) + ENSO (topic) / “El Niño” (topic) / “La Niña” (topic). Acessado em 15/05/2013 em www.webofknowledge.com). Sendo assim, este trabalho é um importante passo inicial no entendimento de como fenômenos climáticos de escala global, que afetam a hidrodinâmica e as condições ambientais (salinidade, substratos, nutrientes), modulam de maneira indireta a diversidade e atividade de bactérias no estuário da Lagoa dos Patos.

No terceiro trabalho, verificou-se que a entrada de água salgada na forma de cunha salina levou a condições ambientais bastante diferentes em curto espaço de tempo, com maiores salinidade, concentrações de nutrientes e seston acontecendo em apenas 24 horas. A variação nas condições hidrológicas tem sido associada a variações no fitoplâncton, um grupo de organismos que apresenta similaridades ecológicas em relação ao bacterioplâncton pelo seu hábito planctônico e faixa de tamanho. Para estes organismos, estudos prévios têm demonstrado que eles são controlados por mudanças na hidrologia derivadas de variações na descarga fluvial e nas variáveis meteorológicas tais como vento, chuva e evaporação. A direção do vento é o principal fator que atua em curtas escalas (horas a semanas), enquanto que em escalas maiores (meses a anos), a descarga fluvial

condiciona a exportação (alta descarga e baixo tempo de retenção) ou a retenção da biomassa fitoplanctônica (baixa descarga, alto tempo de retenção) (Fujita & Odebrecht 2007, Abreu et al. 2010).

Desta forma, conforme hipotetizado por Cesar (2002), é possível que haja um efeito direto da hidrodinâmica sobre a exportação ou a retenção da biomassa bacteriana da mesma forma que observado para o fitoplâncton. Entretanto, esta hipótese precisa ser testada em futuros trabalhos.

4.3 Efeito da salinidade sobre CCB e PFCB

O papel primordial da salinidade na estruturação da composição da comunidade bacteriana foi confirmado pelo claro padrão de diferenciação de comunidades bacterianas em diferentes faixas de salinidades (conforme observado nos três trabalhos, APÊNDICES 1, 2 e 3) e sua alta estabilidade dentro de cada faixa de salinidade (APÊNDICE 1).

De fato, é bem conhecido que há diferenças marcantes nas comunidades bacterianas de acordo com a salinidade (Glöckner *et al*. 2009; Yokokawa *et al*. 2004; Campbell & Kirchman 2012), havendo similaridades mesmo entre ambientes muito distintos em função deste fator, como por exemplo fontes hidrotermais e marismas (salinos) do que em relação a solos de florestas, lagos e desertos (não salinos) (Lozupone & Knight 2007). O estabelecimento de ótimos de crescimento bacteriano por faixa de salinidade pode ser um dos mecanismos desta diferenciação (Langenheder *et al*. 2003).

Neste sentido, os resultados dos três estudos corroboram o padrão estabelecido de diferenciação de comunidades bacterianas pela salinidade,,o

que é especialmente crítico para a faixa mesohalina. Devido à alta dinâmica do estuário, este resultado indica que as taxas de duplicação são suficientemente altas e permitem que a comunidade estabeleça uma nova composição rapidamente em resposta às perturbações ambientais. Em estuários tem sido reportadas taxas de duplicação < 1,0 dia^{-1} (Crump et al. 2004) e especificamente para a Lagoa dos Patos pode chegar a 2,47 dia^{-1} (calculado a partir de Abreu et al. 1992), de forma que as taxas de duplicação podem justificar o padrão observado, mesmo sob condições de alta variabilidade nas condições ambientais.

A salinidade também esteve associada a mudanças na atividade bacteriana, indicando uma diminuição de atividade (menor riqueza de substratos oxidados) em águas mais salinas (APÊNDICES 1 e 2). Este resultado, corroborado por estudos prévios em outros estuários (Trousselier et al. 2002, del Giorgio & Bouvier 2002, Cunha et al. 2000), levaram à conclusão de que a atividade bacteriana diminui em águas mais salinas. Cabe ressaltar que nos dois primeiros artigos a concentração de nutrientes e substratos sempre foi mais alta nas águas menos salinas, um padrão que é esperado em estuários em geral pelo aporte das bacias de drenagem. No entanto, no terceiro artigo uma situação interessante ocorreu: houve a entrada de água mais salina no estuário e ao mesmo tempo mais rica em nutrientes e o padrão se inverteu, com uma maior riqueza de substratos oxidados em águas mais salinas. Isto nos levou a questionar o papel primário da salinidade sobre a atividade bacteriana. Os resultados parecem indicar que ambas as comunidades (água doce e salgada) podem ser igualmente ativas, dependendo das condições ambientais, especialmente a disponibilidade de nutrientes, a que estão submetidas. A

conclusão de que comunidades de água salgada sempre apresentam menor atividade pode ser, desta forma, resultante do padrão comumente encontrado em estuários onde a água salgada é sempre mais pobre em nutrientes inorgânicos dissolvidos e substratos orgânicos. Desta forma, os resultados apresentados no terceiro artigo indicam que o paradigma de que a atividade bacteriana diminui em direção à região costeira dever ser revisto.

Assim, a composição da comunidade bacteriana e a atividade parecem responder primariamente a diferentes fatores: a CCB à salinidade e a atividade aos nutrientes e substratos.

4.4 Efeito de nutrientes inorgânicos, substratos orgânicos, clorofila *a* e seston sobre CCB e PFCB

A dinâmica dos nutrientes nos estuários é bastante complexa, pois além de possuírem origens múltiplas (pontuais e difusas), sofrem ainda muitas transformações químicas, físicas e biológicas ao longo do gradiente salino (National Estuarine Experts Workgroup 2010). Uma característica normalmente encontrada em muitos estuários, porém, é que o suprimento de nutrientes está fortemente associado à entrada de água doce (National Estuarine Experts Workgroup 2010), havendo assim covariação da salinidade com nutrientes (menores concentrações em águas com maior salinidade, conforme observado nos dois primeiros trabalhos - APÊNDICES 1 E 2). Desta forma, em muitos casos é impossível separar o efeito individual destes dois fatores sobre as bactérias.

Em todos os trabalhos (APÊNDICES 1, 2 E 3) foram encontradas relações significativas entre CCB e PFCB com nutrientes inorgânicos (NH_4^+, NO_2^-, NO_3^-, PO_4^{-3}) e substâncias de baixo peso molecular. No terceiro trabalho (APÊNDICE 3) houve ainda relações com seston e clorofila *a*, indicando que estas variáveis têm influência sobre os padrões de diversidade e atividade encontrados, o que encontra suporte na literatura. É importante destacar que nos dois primeiros trabalhos (APÊNDICES 1 e 2), o padrão encontrado foi o de águas mais salinas e mais pobres em nutrientes, não sendo possível separar o efeito destes dois fatores. No terceiro trabalho (APÊNDICE 3), porém, a ocorrência de entrada de cunha salina associada a maiores concentrações de nutrientes permitiu o desacoplamento do efeito dos nutrientes e da salinidade, sugerindo que a atividade bacteriana é uma resposta primária a nutrientes e substratos e apenas secundária à salinidade.

As bactérias absorvem nutrientes inorgânicos dissolvidos (Kirchman 1994), tendo sido demonstrado que a suplementação experimental de nutrientes pode levar ao desenvolvimento de comunidades de bactérias distintas em relação a controles não suplementados (Schäfer *et al*. 2001; Leflaive *et al*. 2008). Sabe-se também que grupos de bactérias são afetados distintamente por diferentes níveis de produtividade primária (Horner-Devine et al. 2003) e podem desenvolver composições de comunidade diferentes quando crescem em presença de carbono orgânico dissolvido de baixo ou alto peso molecular, mesmo derivando de um mesmo inóculo (Covert & Moran 2001). O seston, por sua vez, pode contribuir diretamente com bactérias aderidas, que podem ser composicionalmente diferentes das bactérias-livres (Jing & Liu 2012). Desta forma, estes fatores podem atuar secundariamente, mas em conjunto com a

salinidade na determinação da composição das comunidades bacterianas na Lagoa dos Patos.

A atividade bacteriana, por sua vez, também pode ser afetada por estes fatores. Tem sido demonstrado experimentalmente que a adição de nutrientes leva a diferentes PFCB (Leflaive et al. 2008). O seston, por sua vez, pode contribuir de forma indireta, pois bactérias aderidas apresentam em geral maior atividade que livres (Anésio et al. 2003), apresentando maior riqueza de substratos oxidados no PFCB (Lyons & Dobbs 2012).

O efeito de gradientes de produtividade e substâncias de baixo peso molecular ainda não foram avaliados sobre a PFCB. É possível que haja co-variação da resposta PFCB com clorofila, em função da dependência do fitoplâncton com relação aos nutrientes. Já as substâncias de alto e baixo peso molecular podem determinar diferentes comunidades bacterianas (Covert & Moran 1996), que se utilizam de diferentes conjuntos de enzimas para sua degradação (Münster & Chróst 1990) e , portanto, podem haver impactos sobre o padrão de decomposição da comunidade bacteriana.

No entanto, Abreu et al. (1992) observaram uma ausência de relação entre bactérias aderidas e seston em coletas semanais durante um ano em uma estação fixa em uma reentrância (saco) do estuário da Lagoa dos Patos (Ilha dos Marinheiros). Esta diferença pode ser devido à menor influência da cunha salina neste local,localizado a aproximadamente 30 km da ponta dos molhes que protegem a barra do Rio Grande, enquanto que o píer da Barra onde foram coletadas as amostras do segundo trabalho encontra-se a 5 km da ponta dos molhes, sofrendo provavelmente maior influência da ressuspensão e a contribuição de bactérias aderidas.

Desta forma, os nutrientes e substratos parecem ser o fator primário influenciando a atividade bacteriana. Os nutrientes inorgânicos e a matéria orgânica que provém das bacias hidrográficas são transportadas através dos estuários e podem ser processadas por diferentes populações de micro-organismos: água doce, salobra e marinha (Trousselier et al. 2002). Desta maneira, as mudanças na atividade bacteriana em resposta às condições ambientais podem determinar diferenças importantes na ciclagem de elementos e outros processos bacterianos ocorrendo na coluna d'água em faixas de salinidade diferentes, associados a períodos longos de dessalinização (El Niño) ou salinização (La NIña) ou ainda a processos de curta escala temporal (entrada de cunha salina), com reflexos para a ecologia do estuário.

4.5 Considerações sobre as limitações dos métodos TTGE e Ecoplate™

Os métodos empregados para o estudo da composição e atividade da comunidade bacteriana apresentam algumas limitações que necessitam ser consideradas.

Para o TTGE, existe a possibilidade de comigração de bandas com sequências diferentes quando há alta similaridade entre elas. Há ainda a possibilidade de formação de mais de uma banda por espécie, quando esta apresenta várias cópias do gene 16S rRNA e estas são polimórficas (Muyzer & Smalla 1998). Ainda, como é uma técnica baseada na amplificação de DNA por PCR, reflete apenas o padrão de espécies com alta frequência de ocorrência (> 1-2%) (Murray *et al.* 1996, Nocker *et al.* 2007) e pode detectar células mortas

ou inativas devido à relativa estabilidade do DNA no ambiente. Mesmo assim, este método apresenta vantagens como rapidez e facilidade de comparação entre comunidades bacterianas e possíveis padrões de co-migração ou formação de bandas múltiplas podem ser investigados posteriormente através de sequenciamento.

Com relação ao ensaio Ecoplate™, este oferece uma resposta interessante do ponto de vista de perfilagem do metabolismo potencial da comunidade bacteriana pela capacidade de teste de vários substratos simultaneamente. Porém, este teste não identifica os substratos (mais) utilizados pelas bactérias *in situ*, pois os substratos nas placas podem não estar presentes ou em concentrações muito menores do que no meio ambiente (Konopka *et al*. 1998, Schultz & Ducklow 2000, Preston-Mafham *et al*. 2002). Além disto, a sub-amostragem pela inoculação de um pequeno volume seleciona algumas espécies (mais abudantes) e ocorrem modificações significativas na composição da comunidade bacteriana ao longo da incubação (Smalla et al. 1998). O surgimento da coloração ainda depende do crescimento de bactérias em altas densidades (> 10^8 células mL^{-1}) (Konopka *et al.* 1998). Desta forma, este ensaio reflete o potencial metabólico das bactérias dominantes e cultiváveis ao invés do potencial metabólico absoluto de toda a comunidade. Mesmo assim, ele é uma função da comunidade original e pode ser utilizado como ferramenta para identificar importantes padrões ecológicos, contanto que estas limitações sejam levadas em conta (Konopka *et al.* 1998, Comte & del Giorgio 2010).

Com relação ao índice Puse proposto, é importante considerar que ele é baseado apenas em dois substratos dentre os 31 presentes nas Ecoplates™ e

por isto pode não refletir necessariamente limitação por fósforo, pois a preferência ou inibição causada por algum destes compostos impacta fortemente o seu cálculo. Vale ressaltar que este índice ainda não foi experimentalmente testado e deve ser interpretado com cautela.

5. CONCLUSÕES

i) *Existe um padrão de recorrência (estabilidade) de espécies de bactérias e suas atividades em diferentes faixas de salinidade?*

A composição da comunidade apresentou diferenças significativas entre as três faixas de salinidade (limnética-oligohalina, mesohalina e polihalina) e estabilidade de composição dentro de cada faixa. A atividade bacteriana foi mais similar entre a faixa mesohalina e polihalina, diferindo estas da limnética-oligohalina. A riqueza de substratos (atividade) oxidados diminuiu nas águas polihalinas.

ii) A *composição das comunidades de bactérias e sua atividade respondem a diferentes escalas temporais e espaciais, inclusive a fenômenos climáticos globais que afetam a hidrodinâmica do estuário, tais como o ENSO?*

A composição da comunidade e a atividade bacteriana estiveram associadas à hidrodinâmica do estuário em larga escala (ENSO), sendo significativamente diferentes entre a fase de baxa salinidade que se seguiu ao El Niño e a fase da alta salinidade durante e após o evento de La Niña. A hidrodinâmica, por sua vez, condicionou variáveis (salinidade, nutrientes, substratos, seston) que atuaram diretamente sobre as bactérias. A riqueza de

substratos oxidados foi menor, enquanto que houve provavelmente maior mineralização de nitrogênio e fósforo e falhas na nitrificação nas águas de maior salinidade.

iii) *Que outros fatores, além da salinidade, afetam a composição da comunidade bacteriana e suas atividades?*

A composição da comunidade e a atividade bacteriana apresentaram variação espacial (em função do gradiente de salinidade e profundidade) e temporal de curta escala (em função da entrada de cunha salina). A composição da comunidade bacteriana foi primariamente estruturada pela salinidade, seguindo o padrão normalmente encontrado em literatura para estuários. A atividade bacteriana foi maior em águas de maior salinidade, que coincidiu com maiores concentrações de nutrientes inorgânicos, sugerindo que esta resposta está relacionada somente secundariamente à salinidade.

6. CONTRIBUIÇÕES E PERSPECTIVAS

Este trabalho permitiu associar variações na composição e atividade da comunidade bacteriana à hidrodinâmica, uma relação já extensamente relatada para outros organismos no estuário e que não era confirmada para bactérias. Este ainda é o primeiro trabalho que relata mudanças na composição e comunidade bacterianas em estuários associada ao fenômeno ENSO.

Foi encontrada uma alta estabilidade da composição da comunidade bacteriana, o que reforça o papel da salinidade na estruturação desta

composição, mesmo em ambientes altamente dinâmicos como o estuário da Lagoa dos Patos.

Outra contribuição foi um aumento do entendimento do padrão de atividade em estuários, que parece estar associado primariamente a nutrientes e substratos e secundariamente à salinidade, diferentemente do encontrado em estudos anteriores.

Mesmo com estes avanços, importantes questões ainda precisam ser estudadas, como por exemplo:

I) A biomassa bacteriana também sofre efeito direto da hidrologia ("washout") similar ao observado para o fitoplâncton?

II) É possível mensurar diretamente diferentes processos ecológicos (e.g. taxas de decomposição, nitrificação, atividade enzimática) ocorrendo em diferentes faixas de salinidade? Como a adição de nutrientes e substratos alteraria estes processos?

III) Qual a composição taxonômica das comunidades de diferentes faixas de salinidade?

IV) Qual o grau de resiliência a perturbações de salinidade de cada comunidade de bactérias?

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APÊNDICES

APÊNDICE 1

Apresentação

Este apêndice refere-se ao artigo intitulado: “Stability of bacterial composition and activity in different salinity waters in the dynamic Patos Lagoon estuary: Evidence from a Lagrangian-like approach”, dos autores Ng Haig They, Lise Maria Holanda Ferreira, Luís Fernando Marins e Paulo Cesar Abreu.

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Stability of bacterial composition and activity in different salinity waters in the dynamic Patos Lagoon estuary: Evidence from a Lagrangian-like approach

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Abstract

We employed a Lagrangian-like sampling design to evaluate the bacterial community composition (BCC - using Temporal Temperature Gel Gradient Electrophoresis), community-level physiological profiles (CLPPs – using the Ecoplate™ assay), and influencing factors in different salinity waters in the highly dynamic Patos Lagoon estuary (southern Brazil) and adjacent coastal zone. Samples were collected monthly by following limnetic-oligohaline (0-1), mesohaline (14-16), and polyhaline (28-31) waters for one year. The BCC was specific for each salinity range, whereas the CLPPs were similar for mesohaline and polyhaline waters, and both were different from the limnetic-oligohaline samples. The limnetic-oligohaline waters displayed an oxidation capacity for almost all organic substrates tested, whereas the mesohaline and polyhaline waters presented lower numbers of oxidized substrates, suggesting that the potential activities of bacteria increased from the polyhaline to oligohaline waters. However, the polyhaline samples showed a higher utilization of some simple carbohydrates, amino acids, and polymers, indicating a shortage of inorganic nutrients (especially nitrogen) and organic substrates in coastal saltwater. The hypothesis of bacterial nitrogen limitation was corroborated by the higher Nuse index (an Ecoplate™-based nitrogen-limitation indicator) in the polyhaline waters, and the importance of NO_2^-, NO_3^-, low-molecular-weight substances and the low-molecular-weight:high-molecular-weight substances ratio, indicated by the canonical correspondence analyses (CCAs). Our results demonstrate the important stability of microbial community composition and potential metabolic activity in the different water salinity ranges, which are independent of the

region and time of the year of sample collection in the estuary. This is a quite unexpected result for a dynamic environment such as the Patos Lagoon estuary.

Introduction

The biggest challenge for aquatic microbial ecology is understanding bacterioplankton patterns, namely the structure-function problem [1]. This is a key issue because bacteria are responsible for a large proportion of key ecosystem processes, such as aerobic and anaerobic respiration and nutrient regeneration [2]. The structure-function problem is particularly critical in estuaries, which are complex ecotones where prediction is hampered by the variable responses of bacteria to changing environmental conditions [3]. Hence, bacterial biodiversity, ecological functions and the manner in which these aspects relate to each other are still poorly understood in estuaries.

In spite of these difficulties, one pattern is well recognized: there are changes in the bacterioplankton community composition (BCC) according to the salinity gradient [4-8]. Bacteria from freshwater and land mix with marine bacteria [5], with the possibility of establishing an "estuarine" biota in between, depending on the water residence time [9]. The characteristics of bacterial metabolism, an important aspect of bacterial functions, display contrasting patterns with increasing salinity, such as increases [10] or decreases in secondary production, respiration and growth efficiency [5,11]. However, one aspect that is still poorly understood is the degree of stability of bacterial diversity and its function in different salinity water masses, particularly in very dynamic estuaries.

Two main sampling approaches have been commonly employed when studying the microbial ecology of estuaries. The Eulerian approach, the most common and operationally easiest method, consists of collecting samples at fixed sampling stations over time or space. However, such a sampling method may obscure patterns of bacterial variation in dynamic environments, especially those under the influence of environmental parameters and with high variations in amplitude and frequency, such as salinity, wind and precipitation. An alternative is a Lagrangian sampling approach, where the same parcel or water mass is tracked and samples are taken from the same water mass [12]. This approach, for example, can be performed by collecting samples aboard a boat drifting down a river in one sampling event. However, for longer term studies, it may be more feasible to follow water masses by measuring a given characteristic of the water (e.g., salinity and/or temperature) and sampling the water whenever that characteristic lies within a previously defined range. This procedure is not strictly a Lagrangian sampling strategy, and, hence, we call it a Lagrangian-like approach.

In this study, we employed this proposed Lagrangian-like approach by collecting monthly samples during one year in three salinity range waters (0-1 - limnetic-oligohaline; 14-16 - mesohaline; and 28-31 - polyhaline), irrespective of the location to determine I) the degree of similarity and stability of the bacterial community composition (BCC) and the community-level physiological profiles (CLPP) among the different salinity range waters, and II) the main factors influencing BCC and CLPP.

Materials and Methods

Site description

The Patos Lagoon is the world's largest choked coastal lagoon. It has ~10^4 km^2, is part of a 2×10^5 km^2 watershed [13] and presents an average discharge of freshwater of approximately 2,000 m^3 s^{-1} [14] (Figure 1). The estuarine region (~10% of the lagoon's area) connects to the Atlantic Ocean through a narrow opening that is 20 km in length, 0.5-3 km wide [15] and 12 m deep [16]. The estuary presents a microtidal regime (± 0.4 m) with a net inflow and outflow of fresh- and saltwater predominantly controlled by wind and precipitation. Winds from the southern sector push coastal water into the estuary, whereas northern winds allow the export of the lagoon's water into the adjacent coastal region [17-18].

Estuarine plume formation is predominantly dependent on the amount of freshwater. The wind is the main controller of the plume behavior with the southwesterly winds contributing to its northward movement, promoting saltwater intrusion into the estuary, whereas the northeasterly winds displace the plume southward and contribute to intensifying the freshwater discharge [14].

Northeasterly winds dominate in spring and summer, whereas the southwesterly winds increase in autumn and winter because of a higher number of cold front system intrusions [18]. This wind pattern leads the salinity to display a seasonal behavior, with the establishment of a succession of regimes along the year: salt wedge, partially stratified (spring), vertically mixed (summer and fall), partially stratified (fall), highly stratified (winter) and no estuary (fluvial regime due to high precipitation, winter to

spring) [17]. However, at any time and location, the salinity may rapidly shift, with significant changes over a period of hours [17-18]. The freshwater discharge also displays a marked seasonality, low in the summer and high at the end of the winter/beginning of the spring [19].

The inner limit of penetration of saltwater varies with the wind and freshwater regime, with the mixing zone transferring to the inner continental shelf during high flood periods. However, during drought periods, the salt penetration can reach the middle of the lagoon, which is more than 150 km far from the lagoon's mouth [16]. In addition to these seasonal patterns, global scale meteorological events periodically affect the Patos Lagoon estuary because it is located in an area under the strong influence of the El Niño Southern Oscillation (ENSO), with high precipitation during El Niño years, whereas during La Niña years, the estuary experiences drought events [20-21].

The coastal region is characterized by an extensive and exposed dissipative sandy beach with a gentle slope [22], predominantly subjected to morphodynamic changes due to meteorological tides during cold front events [23]. These events are associated with mud deposition from the shore face to the foreshore and surf zone, which substantially reduces wave energy and increases shoreline accretion. After cold events, the mud is slowly removed at rates that depend on local hydrodynamics [24].

(Figure 1)

Sampling design and water quality

Sampling was performed monthly during one year between June 2010 and May 2011 in the lower estuary and coastal zones of the Patos Lagoon (Figure 1). We measured the salinity at several points every month and collected samples irrespective of location whenever the salinity was within one of three different salinity ranges: 0-1 - limnetic-oligohaline; 14-16 - mesohaline and 28-31 - polyhaline (Figure 1). Salinity and temperature were measured in the field using an Atago S/Mill-E refractometer and a Labortherm mercury thermometer. Surface water was collected with the help of a plastic bucket. One liter was pre-filtered through a 5.0-µm mesh plankton sieve to exclude most protozoans and metazoans, stored in autoclaved (121°C, 30 minutes) borosilicate bottles, and maintained on ice until filtration in the laboratory. In the laboratory, the pH (Digimed) was measured in an aliquot of the pre-filtered water. Another aliquot (250 mL) was vacuum-filtered through 0.2 µm, 47 mm of diameter, polycarbonate filters to concentrate the bacteria. In an aliquot of the filtrate, the relative concentration of low- (LMW) and high-molecular-weight (HMW) substances and the water color were measured at 250, 365 and 430 nm, respectively (UV-VIS CARY spectrophotometer) [25]. The 250:365 nm ratio was calculated as a gross estimate of the ratio of low- to high-molecular-weight substances (LMW:HMW ratio) [26-27]. Another aliquot (250 mL) was stored in polyethylene flasks at -20°C for colorimetric analyses of NH_4^+ [28], NO_2^-, NO_3^- and PO_4^{-3} [29].

Bacterial community composition (BCC)

Bacterial diversity was determined using the fingerprinting technique temporal temperature gradient gel electrophoresis (TTGE). Polycarbonate filters containing the

concentrated bacteria were stored in sterile microtubes at -20°C. Bacterial DNA was extracted using the commercial kit QIamp DNA Stool (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations (cell lysis at 95°C). The DNA quality was examined by 1% agarose gel electrophoresis.

Nested-PCR was performed using the primers 11F (5'-GTTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') [30], which amplify almost the entire bacterial 16S gene at the first step, and the primers GC-338F (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG - 3') and 518R (5'- ATT ACC GCG GCT GCT GG - 3') [31], which amplify around 180 bp of the V3 hypervariable region within the amplicons of the first step of the PCR. The first step was performed under the following conditions: the final concentrations were 1× Reaction Buffer (initial 10×: 20 mM Tris-HCl (pH 8.0), 40 mM NaCl, 2 mM sodium phosphate, 0.1 mM EDTA, 50% (v/v) glycerol), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer, 20 ng μL^{-1} of BSA, 0.1 µL of Platinum® Taq DNA polymerase (5 U μL^{-1}, Invitrogen) and 1.0 µL of the PCR product containing the first step amplicons in a 12.5-µL reaction. The PCR conditions were as follows: 5 minutes at 94°C; 30 cycles of 5 minutes at 94°C, 1 minute at 52°C and 1 minute and 30 s at 72°C; and a final extension step of 10 minutes at 72°C.

The second step was conducted at a final concentration as follows: 1× amplification buffer (initial 10×: 20 mM Tris-HCl (pH 8.0), 12 mM $MgSO_4$, 40 mM KCl, 1 mM DTT, 0.1 mM EDTA, 50% (v/v) glycerol), 0.3 mM of each dNTP, 0.3 µM of each primer, 1.0 µL of Pfx 50™ DNA polymerase (5 U μL^{-1}, Invitrogen) and 2.0 µL of template DNA in a total volume of 50 µL. The PCR was conducted for 5 minutes at 94°C; 20 touchdown cycles [32] of 1 minute at 94°C, 1 minute starting at 65°C and

ending at 55°C (decreasing 0.5°C each cycle), and 1 minute at 68°C; 20 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 68°C; and 30 minutes at 68°C as a final extension step to minimize artifactual bands [33]. The sizes of the PCR products were validated by 1% agarose gel electrophoresis and quantified using a Quant-It™ dsDNA broad range assay kit (Invitrogen).

TTGE was run using the DCode system (BioRad) based on the manufacturer's recommendations with several modifications that were empirically tested: 14% polyacrylamide gels (37.5:1 acrylamide:bis-acrylamide), 7 M urea and 1.25× TAE buffer. The gels were polymerized with 50 µL of TEMED and 100 µL of 10% APS for 1.5-2 h and submitted to a pre-run at 200 V for approximately 30 minutes. Samples (~600 ng of PCR amplified DNA) were loaded in a proportion of 1:1 (sample:gel) loading dye (70% glycerol, 0.05% bromophenol blue, 0.05% xylenocyanol). The run was set to 66.0-69.7°C, 0.2°C h^{-1} at 68 V [34]. The gels were stained in 1.25× TAE buffer containing 1 µg $µL^{-1}$ of ethidium bromide for 15 minutes and destained in 1.25× TAE buffer for 15 minutes. The gels were photographed using an UV transillumination with T1201 Sigma/Ultra Lum Ultra Cam Digital Imaging equipment coupled to a Power Shot A620 Canon Camera. Calibration and alignment among the gels were performed with the help of an artificial standard made from a mix of 6 bacterial isolates acquired previously in the estuary. This procedure was based on Tourlomousis *et al.* [35] but modified because we only used one standard, which is similar to the "clone ladder" described by Tourlomousis *et al.* [35]. For this procedure, approximately 100 ng of nested PCR-amplified DNA (the same manner as for the samples) from each bacterial isolate was mixed together, and the mix was applied before and after the samples in each gel by following the previous procedure.

Band detection and among-gel alignments were performed using the software Gel Analyzer 2010a [36] after rolling ball background subtraction of the images. Bands were detected automatically and then manually corrected under supervision. The procedure was based on Tourlomousis *et al.* [35] with modifications, i.e., we performed a Rf calibration (reference values, a measurement of band position along the lane relative to its total length) of the bands' positions using the reference bands from the isolate mix standard to anchor smooth curves that followed the same Rf values across different gel images. This procedure allows for the correction of gel distortion and allows within- and among-gel alignments [35]. In contrast to Tourlomousis *et al*. [35], we did not extract densitometric curves (continuous fluorescence profiles) but rather matrices of discrete band positions (Rf values) with respective band raw volumes (peak area × peak height), which are generated as a default by Gel Analyzer [36]. Instead of using Matlab, band matching was performed using R 2.15.0 [37], employing a cluster-based peak alignment algorithm [38] and a cut-off of 0.01 (the smallest distance at which two bands are considered the same). Output matrices were converted to presence/absence matrices and used in the subsequent statistical analyses.

Community-level physiological profiles (CLPP)

CLPPs were characterized using microtiter plates, Ecoplate™ (Biolog), which indicate the potential oxidation of 31 organic substrates (Table 1) through the redox indicator tetrazolium violet. The original Biolog GN Microplates were designed for microbial identification, and the Ecoplates were derived from Biolog plates as a selection for ecologically relevant substrates. Therefore, Ecoplates are commercial plates, and the set of substrates are standard. The complete list of substrates and their general

characteristics can be found in Table 1. The pre-filtered (5 µm) water taken from the three salinity ranges was inoculated onto the plates (150 µL in each well), and the plates were incubated for seven days at 20°C in the dark. The time between sampling and the start of the incubation varied from 2-3 h. After the incubation, the wells were individually homogenized using an automatic pipettor and read at 595 nm using a plate reader (TP Reader NM Thermoplate). The first sampling month (June) was read using a Bio-Tek ELx 800 plate reader at 630 nm and corrected using an absorbance curve of 595 nm × 630 nm. For this correction, one plate from July was read using the two plate readers, and a curve was constructed using linear regression (R^2 = 0.9757; Abs595 = 1.1145[Abs630] - 0.0132) and applied to correct the June plate readings.

The readings were subtracted from the reference well (redox dye only), and the negative values were considered zero. These corrected readings were then divided by the AWCD (average well color development, the average of all corrected readings of each plate) to normalize for differences in initial bacterial densities among the samples [39].

To test the differences among the salinity range samples for the bacterial utilization of nitrogen-based organic substrates, the Nuse index was calculated based on the consumption pattern of nitrogen-based Ecoplate™ substrates (Tables 1 and 2). This index was calculated as the sum of the absorbances of the nitrogen-based compounds over the total absorbances of all compounds for a given plate after correction for the control wells [40]. This index is negatively correlated to the DIN concentration in environmental samples, and increases in the index are a reliable indicator of nitrogen limitation [40].

(Table 1)

Statistical data analyses

Two cluster analyses were performed using UPGMA (unweighted pair group method with arithmetic mean) for I) BCC with Bray-Curtis dissimilarity (presence/absence) after the exclusion of bands with a frequency <10%, and II) CLPP with the correlation dissimilarity (1-correlation) based on AWCD (average well color development) transformed data [41]. The consistency of the salinity range groups was tested using ANOSIM (1,000 permutations) in PAST 2.14 [42].

Canonical correspondence analyses (CCAs) were run to verify the explanatory power of abiotic variables (temperature, pH, NH_4^+, NO_2^-, NO_3^-, PO_4^{-3}, LMW substances and LMW:HMW substances ratio) on BCC and CLPP. Water color was highly correlated with HMW substances ($r = 0.96$) and was hence excluded from the analysis. For BCC, bands with a frequency of occurrence <10% were excluded. The variables were tested for variance inflation factors (VIFs), and only variables with VIFs <10 were included [43]. The global significance of the CCAs and the significance of the individual axes were tested by permutation analysis ($\alpha = 0.05$, 1,000 permutations). The whole procedure was based on Borcard *et al.* [43] using R 2.15.0 [37] and the packages Vegan [44] and BiodiversityR [45]. For the determination of the explanatory variables with highest loading by axis, we used an arbitrary criterion, considering those with scores above the median for each CCA important. The median was calculated from a set of absolute values of scores for all explanatory variables. This set was chosen by taking the highest score across all significant canonical axes for each explanatory variable.

All calculations (except for ANOSIM) and graphical displays were performed in R 2.15.0 [37].

Results

In total, 24 samples were collected. Because we could not locate the three salinity ranges for all months, the number of samples was uneven for each range. Nine, 5 and 10 samples were collected from the limnetic-oligohaline, mesohaline and polyhaline waters, respectively.

Table 2 summarizes the main nutrients and environmental variables measured for each salinity range. In general, there was a gradient of higher values of environmental variables for the low salinity waters, decreasing towards the higher salinity waters. The limnetic-oligohaline samples had the highest values for NH_4^+, PO_4^{-3}, LMW substances, and LMW:HMW ratio, the mesohaline waters were intermediate, and the polyhaline waters were the lowest. The limnetic-oligohaline waters also had the highest values for NO_2^-, NO_3^-, water color, and HMW substances, the polyhaline waters were intermediate, and the mesohaline waters were the lowest (Table 2).

(Table 2)

A total of 59 ribotypes (band positions) were identified based on the TTGE, 45 with a frequency >10%. Almost 25% of the bands were shared among all salinity ranges, but some of them were exclusive to each range. The polyhaline range was the one with highest number of exclusive band positions, whereas the limnetic-oligohaline and mesohaline samples had the same number of exclusive bands. Most of the bands were shared between two out of the three salinity ranges; for these the highest number of shared bands was between limnetic-oligohaline and polyhaline samples (Figure 2; Table 3).

(***Table 3***)

The BCC cluster was composed of two main groups separated at ~80% Bray-Curtis dissimilarity: one containing polyhaline samples and the other limnetic-oligohaline and mesohaline water samples. The mesohaline samples split from the limnetic-olihogaline samples at approximately 75% dissimilarity (Figure 2).

(Figure 2)

Although the CLPP from June (0-1) was not available because the plate was accidentally lost, the CLPP cluster also separated the samples by salinity, except for May in the limnetic-oligohaline range and June in the polyhaline range. For all other months, two major groups were composed at ~80% of the correlation dissimilarity by I) polyhaline and mesohaline samples and II) all limnetic-oligohaline samples plus 2 polyhaline samples (March and May, Figure 3).

Considering the responses of the different salinity ranges, there was a gradient of substrate utilization from the limnetic-oligohaline to polyhaline waters. The limnetic-oligohaline samples showed a pattern of low (< 2 times the absorbance divided by the AWCD) consumption for almost all substrates, whereas the mesohaline and polyhaline samples showed a pattern of consumption of even less substrates. However, some of the substrates were more heavily (> 4 times the absorbance divided by the AWCD) utilized, such as simple carbohydrates (D-mannitol, N-acetyl-D-glucosamine and D-cellobiose), amino acids (L-serine, L-threonine and glycyl-L-glutamic acid) and polymers (α-cyclodextrin and glycogen) (Figure 3).

(Figure 3)

The ANOSIM confirmed the consistency of the sample groups based on salinity. For BCC, limnetic-oligohaline, mesohaline and polyhaline groups all differed among them. For CLPP, there was no significant difference between the mesohaline and polyhaline samples, which, in turn, were both different from the limnetic-oligohaline samples (Table 4).

(Table 4)

The sampling sites in the CCA of the BCC were, in general, more spread out, whereas, in the CCA of the CLPP, the sites were more tightly arranged near the origin of the canonical axes. The CCA for the BCC was marginally significant (*pseudo*-F = 1.17 p = 0.07), and the constraining variables explained 38.39% of the total inertia (unadjusted R^2). The first (10.17%) and second (7.43%) canonical axes accounted for 17.6% of the total constrained variance explained. Up to 2 axes were significant at $\alpha = 0.05$: CCA1: *pseudo*-F = 2.48; p = 0.003, and CCA2: *pseudo*-F = 1.81, p = 0.03. The variables with the highest contribution per axis in decreasing importance (higher loadings) were as follows: axis 1: LMW:HMW ratio and NH_4^+; axis 2: NO_2^-, NO_3^-, LMW substances, PO_4^{-3}, temperature, and pH.

According to the arbitrary median criterion, the important variables were, in decreasing order: NO_2^-, NO_3^-, LMW:HMW ratio, and LMW substances (Figure 4A).

The CCAs of the CLPP data were also marginally significant (*pseudo*-F = 1.34, p = 0.084). The constraining variables accounted for 43.31% of the total inertia (unadjusted

R^2), with the first (20.31%) and second (7.41%) canonical axes accounting for 27.72% of the total constrained variance explained. Only the first axis was significant at α = 0.05: CCA1: *pseudo*-F = 5.01; p = 0.04. The variables with the highest contribution per axis in decreasing importance were as follows: axis 1: pH, LMW substances, NO_2^-, NH_4^+, temperature, PO_4^{-3}, LMW:HMW ratio, and NO_3^-. The important variables according to the arbitrary median criterion were, in decreasing order: pH, LMW substances, LMW:HMW ratio, and NO_2^- (Figure 4B).

(Figure 4)

Discussion

In contrast to previous studies that have addressed bacterial compositions and activities in estuaries carrying out samplings at fixed stations (e.g., 4, 11, 46-49), we established salinity ranges as sampling units independent of space and time. This approach allowed us to identify some important patterns about the bacterial composition and activity in different salinity waters. For example, a consistent and specific BCC was found for each salinity range no matter the location or the time of year. The clustering of the BCC analysis allowed us to identify discrete communities inhabiting waters with different salinities, which suggests that the stability is high despite the fact that the Patos Lagoon estuary is a very dynamic ecosystem [18, 21]. Still, the closer clustering of subsequent months found in our study indicates the presence of a seasonal component, which is a common feature in estuarine and coastal environments for bacterial diversity and metabolism [30, 50-51].

The closer clustering of the BCC of the limnetic-oligohaline and mesohaline samples suggests that there is a more important input of cells to the mixing zone from freshwater than from saltwater. However, most of the bands were shared between limnetic-oligohaline and polyhaline samples, not between limnetic-oligohaline and mesohaline samples. The closer clustering between limnetic-oligohaline and mesohaline samples can be justified by their higher number of exclusive shared bands in comparison with the shared bands between mesohaline and polyhaline samples, and also the high number of exclusive band positions found for polyhaline range.

Nevertheless, it is not possible to conclude about resident and transient bacteria in each salinity range because TTGE does not distinguish among living, dead or inactive cells. Many freshwater bacteria may arrive to this mixing zone in large enough numbers to be detected but do not survive, and/or they remain inactive. In spite of the useful ecological information extracted, TTGE presents the same limitations of any PCR-based technique and does not quantitatively reflect the original community but rather the PCR product pool [52], and predominant species that contribute >1-2% to the sample [46, 52]. Additional investigations with (partial) genome sequencing are necessary to confirm the patterns of bacterial diversity revealed in this study.

If we consider the higher richness of substrates oxidized in the limnetic-oligohaline samples as an indication of a higher potential for substrates degradation compared to higher salinity samples, there is a decrease in the potential bacterial activity from fresh- to saltwater, as reported elsewhere [10]. Decreases in bacterial activity along salinity gradients have also been measured using culture-independent approaches, for example, lower thymidine and leucine incorporation [5, 11], lower percentages of respiring (CTC+), membrane intact and high DNA bacteria [4] along salinity gradients. These

studies might explain the finding that the mesohaline samples, in spite of a similar BCC to the limnetic-oligohaline samples, displayed a CLPP similar to the polyhaline samples, suggesting that many cells of limnetic-oligohaline origin detected by TTGE may be dead or inactive in the mesohaline range. Moreover, this similar result found in the Patos Lagoon estuary suggests that the decrease in bacterial activity towards saltwater may be a common feature of many estuaries.

Despite the lower number of substrates oxidized in the saltwater, there was a tendency towards a higher potential consumption of some carbohydrates (D-mannitol, N-acetyl-D-glucosamine and D-cellobiose), amino acids (L-serine, L-threonine and glycyl-L-glutamic acid) and polymers (α-cyclodextrin and glycogen) in the polyhaline samples, which suggests that saltwater bacteria may be in need for more carbon and nitrogen than freshwater bacteria. This result was confirmed by the higher Nuse index found in the polyhaline waters, which is in accordance with previous findings that there is a strong trophic gradient along the Patos Lagoon estuary, with decreasing chlorophyll *a*, NO_3^-, PO_4^{-3}, bacterial production and bacterial density towards coastal water [53]. This gradient can be so strong that the NO_3^- and PO_4^{-3} present in low salinity waters can be completely depleted before reaching the mixing zone of the Patos Lagoon estuary [54]. A very similar result (a decrease in substrate consumption richness and increase in the consumption of amino acids and carboxylic acid towards higher salinity waters) was found in an Indian estuary by Thottathil *et* al. [55], indicating that nutrient limitation in saltwater can shift the physiological status of bacteria. In contrast, Sala *et al*. [56] found a lower number of Ecoplate™ substrates consumed in the higher nutrient waters of Spanish harbors when compared to the more oligotrophic coastal waters. Those authors attributed this result to a more constant environment in terms of hydrodynamic and

organic matter supply in harbors in comparison to the changing inputs of nutrients in coastal waters, which likely forces bacteria to display a higher plasticity in their metabolic pathways. In the Patos Lagoon estuary, the more dynamic nature of the estuary (in terms of salinity and nutrient input variation when compared to coastal waters) likely forces the bacteria, in spite of the higher nutrient content of the limnetic-oligohaline waters, to adapt to variable pulses of substrates.

The characterization of functional profiles in the form of substrate utilization, as in the Ecoplate™, has been under strong criticism [57-59]. As pointed out by Schultz and Ducklow [10] and Konopka *et al.* [58], the substrate utilization profiles of the plates cannot identify the elements most utilized by bacteria because the substrates in this device may be absent or in much lower concentrations in the environment. Moreover, significant changes in community structure occur during the course of the incubation [57], and color development depends on the bacteria being able to reach high densities ($>10^8$ cells mL^{-1}) [58]. Thus, these results reflect the metabolic potential of culturable dominant bacteria rather than the bacterial community's absolute metabolic capability. For this reason, we can only interpret this information on a comparative basis. Nevertheless, we could extract important ecological inferences that have been observed in other studies using methodologically independent approaches. This may be a first step for the identification of different processes (e.g., amino acid consumption and chitin and cellulose degradation) occurring predominantly or exclusively under different salinity ranges, a subject that requires further investigation.

The cluster analyses provided us with some insights with regards to controlling factors. Salinity could not be formally included in the CCAs because of the type of sampling design; however, its effect was evident because of the large clusters of samples, which

clustered according to salinity range more clearly for BCC but also for CLPP to a lesser extent. Another effect was the temperature, as indicated by the smaller clusters formed by adjacent months. Together, these two environmental factors have been extensively reported to be the main drivers of bacterial community composition in many estuaries [31, 60]. Salinity is the major driver structuring bacterial diversity [61], overcoming other factors such as geographic distance [62]. As a strong driver of bacterial community composition, temperature is known to control the seasonal succession of bacterioplankton in most aquatic ecosystems [31, 63] and coastal zones [64].

The CCAs of the BCC and CLPP were marginally significant, suggesting that many of the samples were not clearly associated with the explanatory variables tested. This result was indicated by their positioning close to the origin of the CCA axes and was more critical for CLPP, which indicates that the functional responses are less constrained by the environmental variables. Hence, it is possible that either I) the explanatory variables tested did not explain a large portion of the BCC and CLPP variation; or II) the explanatory variables do explain a significant fraction of the variation in BCC and CLPP, but these variables display some lagging response to the explanatory variables; III) other important variables were not included in the analysis (e.g., chlorophyll *a*, dissolved oxygen, organic nitrogen and phosphorus); or IV) the metabolic plasticity of bacteria adds significant noise to the analysis. Given the highly dynamic nature of the estuary, any of these hypotheses may be true, as well as any combination of them.

Taking into consideration that both CCAs were marginally significant, we discuss the effects of the most important environmental variables. Both analyses indicated a significant effect of nitrogen-based compounds (NO_2^- and NO_3^-), pH, LMW substances,

and the LMW:HMW ratio. The specific responses of the nitrogen-based compounds may be related to their assimilation [65] and nitrification and denitrification processes, which are controled by pH [66]. In addition, a gradient of nitrogen limitation, indicated by the Nuse index [40], was located from the polyhaline to limnetic-oligohaline waters. Low-molecular compounds availability are important because they are more likely to be readily taken up by bacteria, since bacterial membrane porins have a size exclusion limit of approximately 600 Da [67], lower than the polymers used in Ecoplates™ (Table 1) and many substrates found in nature like cellulose, chitin and proteins.

Given that for any point in the estuary, the salinity shifts drastically over a period of hours [17-18], even when samples were taken at the same location in consecutive months, those locations must have subsequently experienced substantial salinity shifts due to water exchanges. Thus, the maintenance of different bacterial community compositions in the Patos Lagoon estuary in different salinity ranges can be true under two conditions I) that there is a consistent differentiation between fresh- and saltwater bacteria in the estuaries, and II) that estuarine bacteria may display doubling times fast enough to cope with the high frequency shifts in salinity.

Bacterial phylogenetic differences between salt- and freshwater are, in fact, consistent, and, in general, there is a shift of dominance from β-, δ-, ε-Proteobacteria and Actinobacteria in freshwater to α-Proteobacteria and γ-Proteobacteria in saltwater [4, 8-9, 31, 48, 68-70).

This high stability of bacterial community in different salinity waters, as found in Patos Lagoon, points to the fact that salinity imposes a strong stabilizing effect on BCC and CLPP, though CLPP to a lesser extent. This fact has important ecological implications

because the specific BBCs and CLPPs found in different salinity ranges in Patos Lagoon may represent structured differences in ecosystem processes and functioning. Moreover, the high stability found for BCC and CLPP were surprising for a very dynamic system such as the Patos Lagoon estuary, which can only be explained by fast bacterial growth rates. In fact, rates of bacterial community doubling of < 1 day have been estimated in estuaries [9, 71], suggesting that the stability of a bacterial community is feasible even at a high frequency variation in salinity.

However, some limitations of the Lagrangian-like approach need to be considered. First, without a fine scale spatial and temporal measurement of salinity, one cannot be sure of the changes that the different water masses underwent before each sampling event. This point is critical for the mesohaline range because, in our case, it could have purely resulted from water movement (true water mass), or, in some months, it could have resulted from low and high salinity water masses mixing. This is a pivotal issue when discussing stability, for example, it implies completely different interpretations in both cases. However, no matter which of the mechanisms prevail (water movement or mixture), it is noteworthy that the mesohaline samples displayed high stabilities in the BCC and CLPP. The interpretation of stability in this case for the BCCs and CLPPs in the polyhaline and limnetic-oligohaline samples was straightforward since water mix does occur in these salinity ranges, but not as strong and frequent as in the mesohaline waters. We suggest that future studies should include a Lagrangian-like approach performed at a higher frequency to better estimate the temporal and spatial changes in the salinity in the sampling area, with special regard given to mesohaline waters.

Our results displayed a clear differentiation between bacterial communities and potential metabolic activities in different salinity ranges, no matter the location and time

of the year, pointing out that the structuring effect of salinity and also inorganic nutrients and organic substrates overcomes the environmental perturbations inherent to this very dynamic ecosystem. Such degree of stability is remarkable, since the monthly sampling frequency was much longer than the doubling times of estuarine bacteria [9] and thus encompassed several generations of bacterial cells and possibly a wide range of environmental conditions.

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Titles and legends to figures

Figure 1: Patos Lagoon estuary and adjacent coastal area. Map source: Google [69]. Modified with the R 2.15.0 packages{ggmap}[70] and {maps} [71].

Figure 2 Cluster analysis of bacterial community composition (BCC) assessed through TTGE in three salinity ranges (limnetic-oligohaline (0-1), mesohaline (14-16) and polyhaline (28-31)) in the Patos Lagoon estuary and adjacent coastal zone between June 2010 and May 2011. The composite photograph was made of lanes reordered from original gels. The exclusive band positions for each salinity range are marked in the image: L-O, limnetic-oligohaline; M, mesohaline; P, polyhaline.

Figure 3. Cluster analysis of Community-Level Physiological Profiles (CLPP) in three salinity ranges (limnetic-oligohaline (0-1), mesohaline (14-16) and polyhaline (28-31)) in the Patos Lagoon estuary and adjacent coastal zone from June 2010 to May 2011. Xi

corresponds to the corrected absorbance at 595 nm of each well divided by the plate's respective AWCD. Classes of substrates are indicated by: (□) carbohydrate, (■) carboxylic acid, (Δ) amino acid, (▲) polimer, (○) phosphorilated chemical, (●) amine, (♦) ester.

Figure 4 Canonical Correspondence Analysis (CCA) of A) bacterial community composition (BCC) and B) Community-Level Physiological Profiles (CLPP) in three salinity ranges (limnetic-oligohaline (0-1, Δ), mesohaline (14-16, ●) and polyhaline (28-31, ■)) in the Patos Lagoon estuary and adjacent coastal zone between June 2010 and May 2011. Sample labels identified by months: Jan, January; Feb, February; Mar, March; Jun, June; Jul, july; Aug, August; Sep, September; Oct, October; Nov, November; Dec, December. Explanatory variables: Temp, temperature; pH, potential of Hydrogen; NH_4^+, ammonium; NO_2^-, nitrite; NO_3^-, nitrate; PO_4^{-3}, ortophosphate; LMW, low-molecular-weight substances; LMW:HMW, low:high-molecular-weight substances ratio.

Tables

Table 1 List of organic substrates found in Ecoplate™ (Biolog) with general chemical characteristics.

Substrate	Chemical group[1]	Molecular Weight[2]	Nutrient Source[3]
Putrescine	amine	88.15	C+N
α-Ketobutyric Acid	carboxylic acid	102.09	C
Pyruvic Acid Methyl Ester	ester	102.09	C
γ-Hydroxybutyric Acid	carboxylic acid	104.10	C
L-Serine	amino acid	105.09	C+N
L-Threonine	amino acid	119.12	C+N
Phenylethyl Amine	amine	121.18	C+N
i-Erythritol	carbohydrate	122.12	C
Itaconic Acid	carboxylic acid	130.10	C
L-Asparagine	amino acid	132.12	C+N
D-Malic Acid	carboxylic acid	134.09	C
2-Hydroxy Benzoic Acid	carboxylic acid	138.12	C
4-Hydroxy Benzoic Acid	carboxylic acid	138.12	C
D-Xylose	carbohydrate	150.13	C
L-Phenylalanine	amino acid	165.19	C+N
D,L-α-Glycerol Phosphate	phosphorylated chemical	172.07	C+P
L-Arginine	amino acid	174.20	C+N
D-Galactonic-Acid γ Lactone	carboxylic acid	178.14	C
α-D-Galactose	carbohydrate	180.16	C
D-Mannitol	carbohydrate	182.17	C
D-Galacturonic Acid	carboxylic acid	194.14	C
β-Methyl-D-Glucoside	carbohydrate	194.18	C
D-Glucosaminic Acid	carboxylic acid	195.17	C+N
Glycyl-L-Glutamic Acid	amino acid	204.18	C+N
N-Acetyl-D-Glucosamine	carbohydrate	221.21	C+N
Glucose-1-Phosphate	phosphorylated chemical	260.14	C+P
D-Cellobiose	carbohydrate	342.30	C
Tween 80	polimer	604.81	C
Tween 40	polimer	620.86	C
Glycogen	polimer	666.58	C
α-Cyclodextrin	polimer	972.84	C

1 - Garland JL, Mills AL (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Appl Environ Microb 57(8):2351-2359.

2 - National Center for Biotechnology Information. PubChem Compound Database. Available at: <http://pubchem.ncbi.nlm.nih.gov>.

3 - Sala MM, Pinhassi J, Gasol JM (2006) Estimation of bacterial use of dissolved organic nitrogen compounds in aquatic ecosystems using Biolog plates. Aquat Microb Ecol 42:1-5.

Table 2 Summary of environmental variables (mean, range, standard deviation (SD) and median) and ratios (Nuse index and LMW:HMW ratio) sampled in the three salinity ranges (limnetic-oligohaline (0-1, n = 9), mesohaline (14-16, n = 5) and polyhaline (28-31, n = 10)) in Patos Lagoon estuary and adjacent coastal zone between June 2010 and May 2011.

Variable	Limnetic-Oligohaline (Salinity 0-1, n=9)			Mesohaline (Salinity 14-16, n=5)			Polyhaline (Salinity 28-31, n=10)		
	Mean (range)	SD	Median	Mean (range)	SD	Median	Mean (range)	SD	Median
Temperature (º C)	19 (11-25)	4	18	23 (18-28)	5	22	21 (16-29)	5	20
pH	7.5 (7.1-8.0)	0.3	7.5	8.5 (7.9-9.9)	0.8	8.2	8.0 (7.7-8.4)	0.2	8.0
NH_4^+ (µM)	5.58 (0-32.16)	10.33	1.58	2.39 (0.72-4.84)	1.59	2.37	2.37 (0-6.06)	1.88	1.66
NO_2^- (µM)	0.87 (0.25-3.56)	1.04	0.40	0.38 (0.15-1.10)	0.40	0.23	0.50 (0.08-1.06)	0.38	0.39
NO_3^- (µM)	3.11 (0-9.56)	4.18	0.67	2.03 (0.19-6.88)	2.85	0.41	2.67 (0.23-7.45)	2.40	1.69
Nuse	0.35 (0.34-0.38)	0.01	0.35	0.30 (0.24-0.33)	0.04	0.32	0.39 (0.25-0.53)	0.09	0.37
PO_4^{-3} (µM)	1.72 (0.36-6.16)	1.84	1.22	0.86 (0.56-1.22)	0.32	0.81	0.80 (0.16-1.82)	0.58	0.69
LMW substances (Abs 250 nm × 10^2)	27.62 (2.91-76.85)	21.94	28.19	12.87 (8.63-23.21)	6.36	8.94	11.14 (3.43-26.38)	8.40	8.13
HMW substances (Abs 365 nm × 10^2)	6.55 (0.37-23.51)	6.79	4.63	3.82 (1.17-10.35)	3.70	2.51	4.18 (0.56-14.53)	4.19	3.31

LMW:HMW ratio (Abs 250:365 nm)	4.9 (1.7-7.9)	1.9	5.2	4.5 (2.2-7.6)	2.2	3.4	3.5 (1.6-6.9)	1.7	3.3
Water Color (Abs 430 nm × 10^2)	3.23 (0.04-14.42)	4.39	1.83	2.46 (0.49-7.35)	2.77	1.57	2.97 (0.17-11.87)	3.54	1.56

Table 3 Total and proportion of exclusive and exclusive shared bands positions detected by TTGE in three salinity ranges (limnetic-oligohaline (0-1), mesohaline (14-16) and polyhaline (28-31)) in Patos Lagoon estuary between June 2010 and May 2011.

	Salinity range	Nº bands	Proportion
Exclusive bands	0-1	4	0.068
	14-16	4	0.068
	28-31	9	0.153
Exclusive shared bands	0-1 × 14-16	8	0.136
	0-1 × 28-31	15	0.254
	14-16 × 28-31	6	0.102
	All	13	0.220
Total		59	1.000

Table 4 One-way ANOSIM among three salinity ranges (limnetic-oligohaline (0-1), mesohaline (14-16) and polyhaline (28-31)) in Patos Lagoon estuary and adjacent coastal zone for BCC (Bacterial Community Composition) and CLPP (Community-Level Physiological Profiles) between June 2010 and May 2011. The values of the ANOSIM R statistic are indicated in bold and the P-values in regular font type.

		Sal 0-1	**Sal 14-16**	**Sal 28-31**	**Global test**
BCC	**Sal 0-1**	-	**0.69***	**0.72***	**R = 0.78***
	Sal 14-16	<0.05*	-	**0.93***	p < 0.05*
	Sal 28-31	< 0.05*	< 0.05*	-	
CLPP	**Sal 0-1**	-	**0.71***	**0.65***	**R = 0.55***
	Sal 14-16	< 0.05*	-	**0.28**	p < 0.05*
	Sal 28-31	<0.05*	0.10	-	

* - statistically significant at $\alpha = 0.05$; Sal, salinity range.

Figures

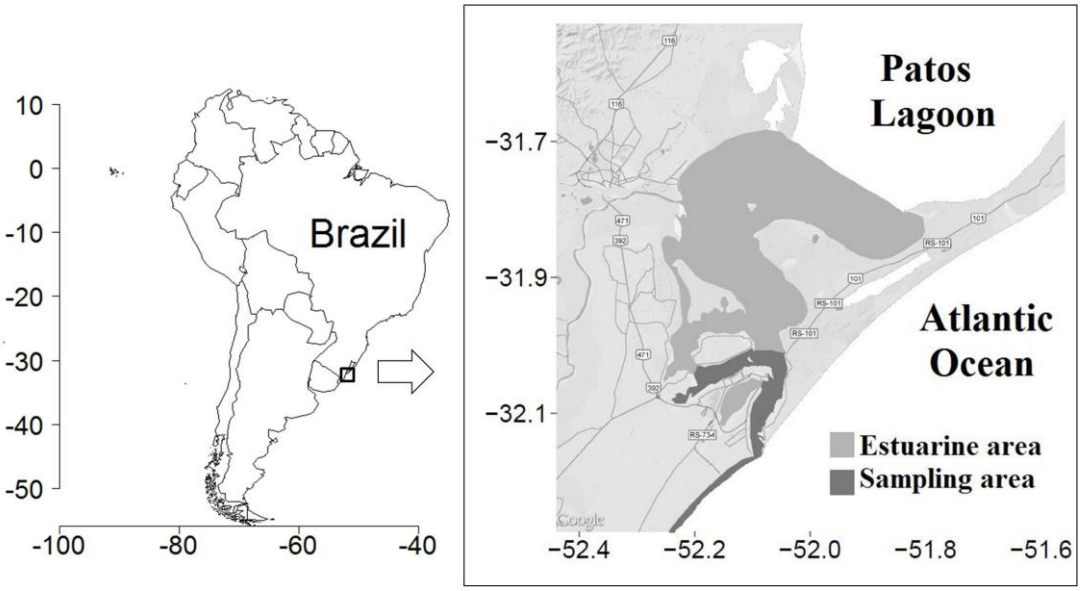

Figure 1

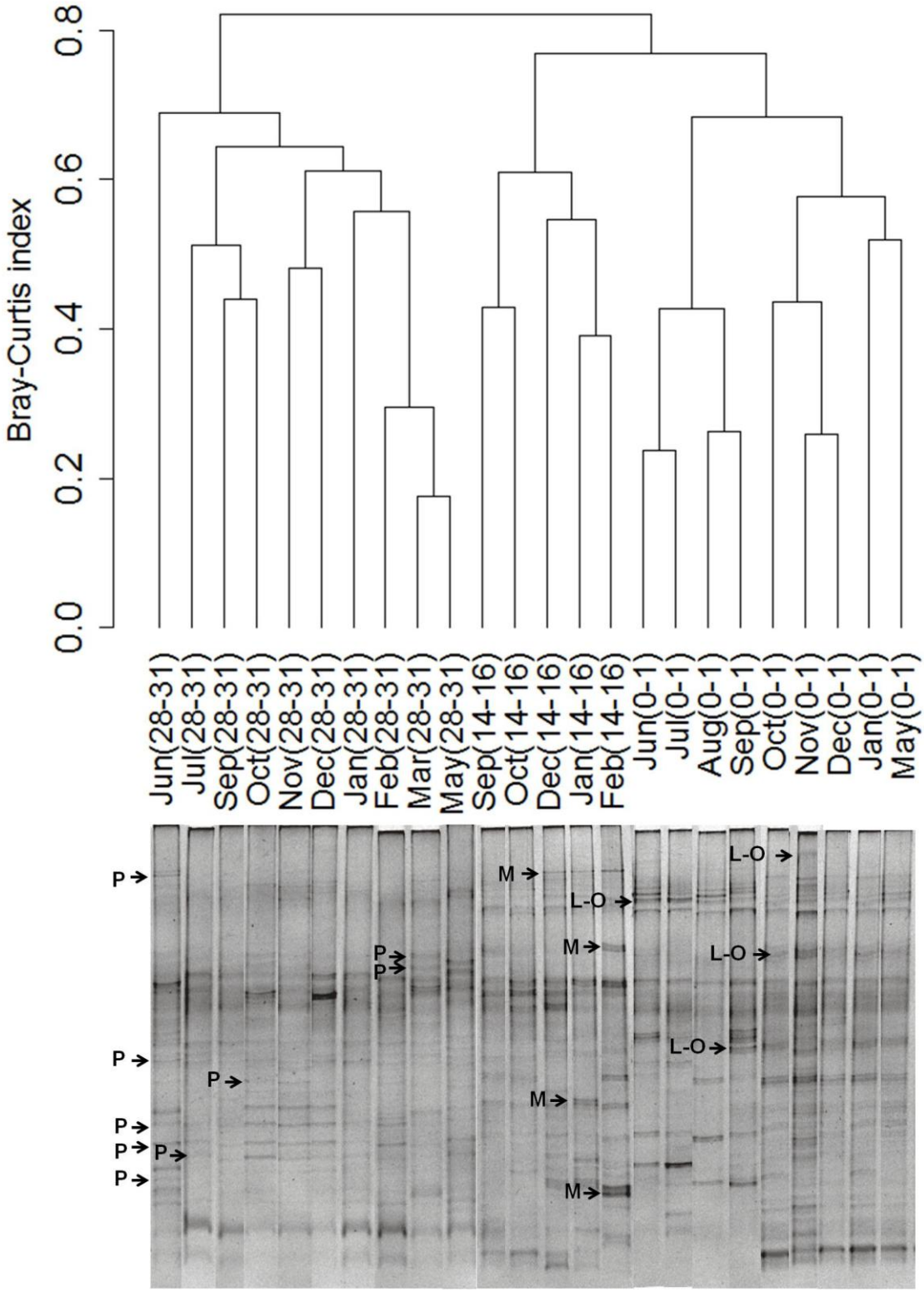
Bray-Curtis index
0.0
0.2
0.4
0.6
0.8
Jun(28-31)
Jul(28-31)
Sep(28-31)
Oct(28-31)
Nov(28-31)
Dec(28-31)
Jan(28-31)
Feb(28-31)
Mar(28-31)
May(28-31)
Sep(14-16)
Oct(14-16)
Dec(14-16)
Jan(14-16)
Feb(14-16)
Jun(0-1)
Jul(0-1)
Aug(0-1)
Sep(0-1)
Oct(0-1)
Nov(0-1)
Dec(0-1)
Jan(0-1)
May(0-1)
P
P
P
P
P
P
P
P
P
M
L-O
M
M
M
L-O
L-O
L-O

Figure 2

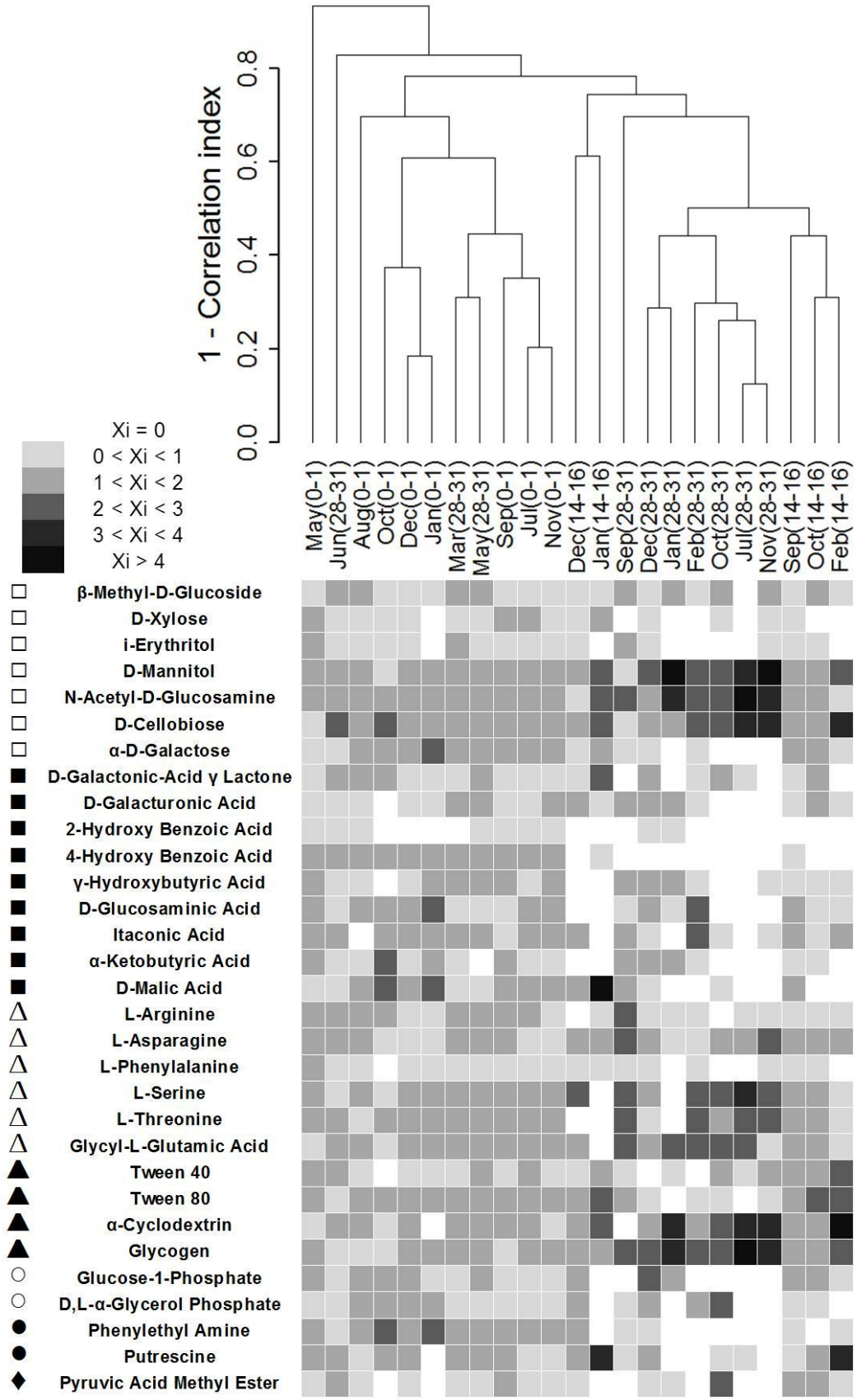
1 - Correlation index
0.0
0.2
0.4
0.6
0.8
May(0-1)
Jun(28-31)
Aug(0-1)
Oct(0-1)
Dec(0-1)
Jan(0-1)
Mar(28-31)
May(28-31)
Sep(0-1)
Jul(0-1)
Nov(0-1)
Dec(14-16)
Jan(14-16)
Sep(28-31)
Dec(28-31)
Jan(28-31)
Feb(28-31)
Oct(28-31)
Jul(28-31)
Nov(28-31)
Sep(14-16)
Oct(14-16)
Feb(14-16)
Xi = 0
0 < Xi < 1
1 < Xi < 2
2 < Xi < 3
3 < Xi < 4
Xi > 4
β-Methyl-D-Glucoside
D-Xylose
i-Erythritol
D-Mannitol
N-Acetyl-D-Glucosamine
D-Cellobiose
α-D-Galactose
D-Galactonic-Acid γ Lactone
D-Galacturonic Acid
2-Hydroxy Benzoic Acid
4-Hydroxy Benzoic Acid
γ-Hydroxybutyric Acid
D-Glucosaminic Acid
Itaconic Acid
α-Ketobutyric Acid
D-Malic Acid
L-Arginine
L-Asparagine
L-Phenylalanine
L-Serine
L-Threonine
Glycyl-L-Glutamic Acid
Tween 40
Tween 80
α-Cyclodextrin
Glycogen
Glucose-1-Phosphate
D,L-α-Glycerol Phosphate
Phenylethyl Amine
Putrescine
Pyruvic Acid Methyl Ester

Figure 2

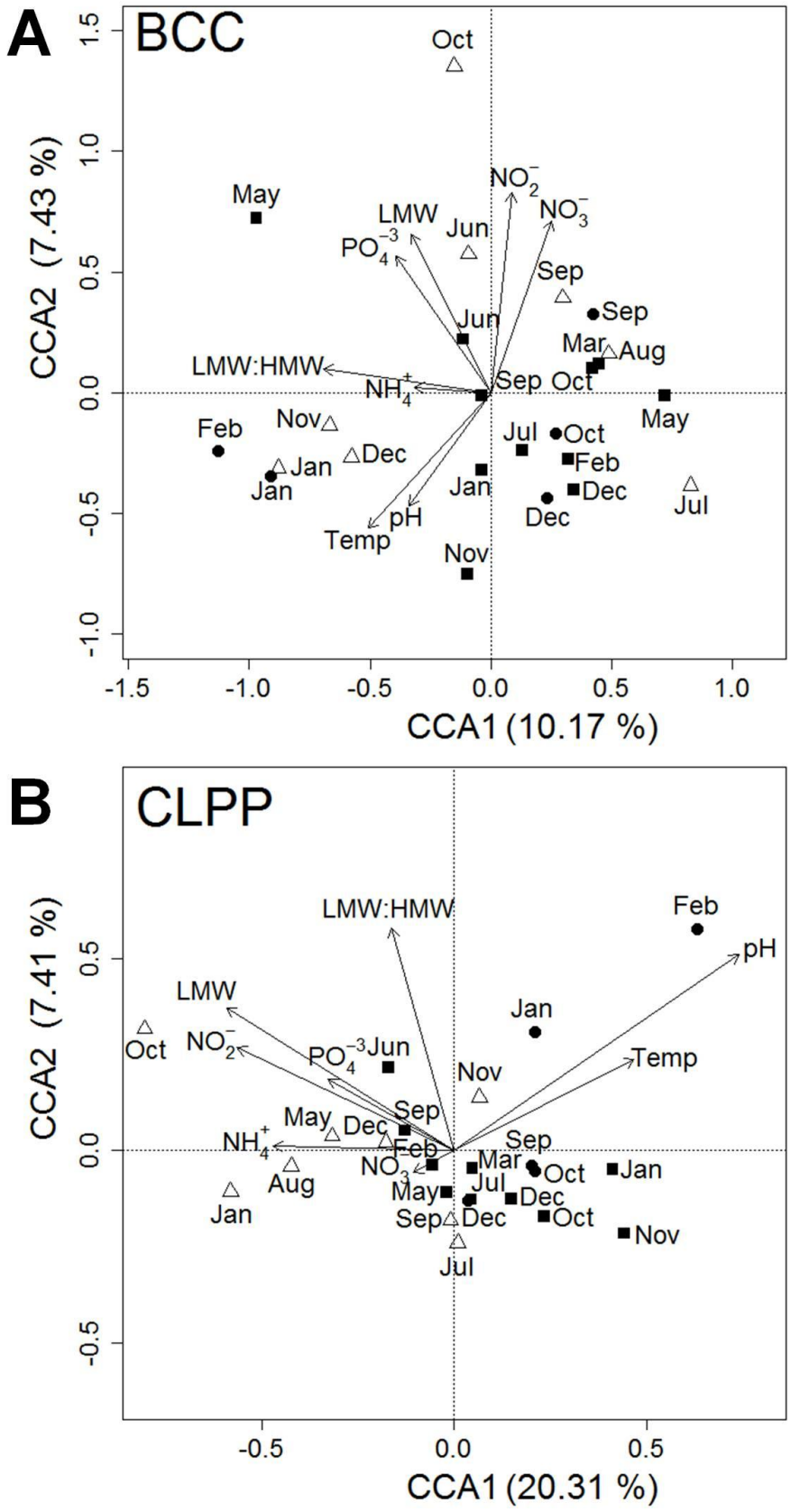
A
BCC
CCA2 (7.43 %)
CCA1 (10.17 %)
Oct
May
LMW
Jun
NO_2^-
NO_3^-
PO_4^{-3}
Sep
Mar
Aug
LMW:HMW
NH_4^+
Feb
Nov
Jul
Dec
Jan
pH
Temp
B
CLPP
CCA2 (7.41 %)
CCA1 (20.31 %)
LMW:HMW
pH
LMW
Oct
NO_2^-
PO_4^{-3}
Jun
Nov
Temp
May
Dec
Sep
NH_4^+
Feb
Mar
NO_3^-
Aug
Jul
Jan
Oct
Nov

Figure 3

APÊNDICE 2

Apresentação

Este apêndice refere-se ao artigo intitulado: “Bacterioplankton phylogenetic and physiological shifts associated to the El Niño Southern Oscillation (ENSO) in the Patos Lagoon estuary”, dos autores Ng Haig They, Lise Maria Holanda Ferreira, Luís Fernando Marins e Paulo Cesar Abreu.

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Bacterioplankton phylogenetic and physiological shifts associated to the El Niño Southern Oscillation (ENSO) in the Patos Lagoon estuary

Ng Haig They[1], Lise Maria Holanda Ferreira[2], Luís Fernando Marins[3] and Paulo Cesar Abreu[4]

Running title: ENSO effect on bacterial communities in Patos Lagoon estuary

Key-words: ECOPLATE™ / nutrient limitation / Temporal Temperature Gradient Electrophoresis / Brazil

Abstract

The Patos Lagoon estuary is a microtidal system strongly regulated by atmospheric forcing, including remote large scale phenomena like El Niño Southern Oscillation (ENSO), which influences precipitation patterns in the region. We investigated whether there was a detectable effect of a transition from a moderate El Niño to a strong La Niña event (June 2010 to May 2011) on the bacterial community composition (BCC), and community level physiological profile (CLPP) along with a set of environmental variables. Two distinct periods were found: a period that followed El Niño, with low salinity, and high concentration of NO_2^-, NO_3^-, PO_4^{-3} and of low molecular weight (LMW) substances, and a period that followed La Niña, when salinity, temperature and transparency increased and nutrients and LMW substances decreased. Bacteria responded to this transition by changing significantly the BCC and CLPP, with increases in consumption of nitrogen- and phosphorus- based organic substrates and accumulation of NH_4^+ and PO_4^{-3} during La Niña phase. This was interpreted as increased rates of mineralization and failures in nitrification. The results suggest a link between ENSO and bacteria, which points out to the role of climate variability on the cycling of organic matter by these microorganisms.

1. Introduction

Most estuaries in the world are dominated to some extent by tides (Wells, 1995), presenting mesotidal (2-4 m) and macrotidal (> 4 m) regimes (Perillo, 1995). In these tide-dominated estuaries, tidal currents exert the main forcing on sediments and salinity distribution (Perillo, 1995). Another type of estuaries, however, is not highly influenced by tides and hence presents a tendency to display microtidal (< 2 m) regimes and to respond mostly to waves, wind (Perillo, 1995) and river discharge (Luettich Jr. *et al.*, 2002).

The Patos Lagoon in southern Brazil, with an area of 10,360 km^2, is the largest choked coastal lagoon in the world, possessing a microtidal (± 0.4 m) regime due to its proximity to an amphidromic point (Soares & Möller Jr., 2001). The estuarine region (900 km^2) in the southern sector has a narrow communication with the ocean. Thus, meteorological factors such as wind, precipitation and evaporation are the main drivers of the Patos Lagoon estuary hydrodynamics, controlling net inflow and outflow of fresh- and saltwater. Winds from the northern sector along with high precipitation contribute to increase the export of lagoon´s water, whereas winds from the southern section along with low precipitation favors the intrusion of coastal water into the estuary (Hartmann & Schettini, 1991; Marques *et al*., 2009; Möller *et al*., 2001).

Because it is located in an area under a strong influence of El Niño Southern Oscillation (ENSO) phenomenon, the Patos Lagoon estuary also suffers the influence of this global scale climatic phenomenon besides seasonal meteorological patterns (Grim *et al*., 1998). El Niño events strengthen the upper-tropospheric subtropical jet currents (a belt of strong westerly winds), favoring the intensification of mesoscale systems and cyclogenesis over southern Brazil and also other countries in South America such as

70 Argentina and Uruguay, causing high precipitation during these periods. La Niña events
71 are associated to the opposite pattern, i.e., weakening of the subtropical jet currents, thus
72 bringing low precipitation and to the region, generating drought events (Grim *et al*.,
73 1998; Garcia *et al*., 2004; Abreu *et al*., 2010). As a consequence, these phenomena are
74 followed by prolonged desalinization (El Niño) or salinization (La Niña) of the estuary,
75 which has been linked to significant biological responses of the distribution of many
76 organisms in this ecosystem.
77 For instance, ENSO has been reported to alter the biomass and community composition
78 of phytoplankton (Abreu *et al*., 2010), the distribution and assemblage composition of
79 fish (Garcia *et al.*, 2001), the distribution of the golden mussel *Limnoperna fortunei*
80 (Capítoli *et al*., 2008) and the recruitment of the seagrass *Ruppia maritima* (Odebrecht
81 *et al*., 2010), as well as the abundance of macrozoobenthic species (Colling *et al.*, 2007;
82 Odebrecht *et al*., 2010), fisheries (Garcia *et al*., 2004) and shrimp catches (Castello &
83 Möller, 1978; Möller *et al*., 2009). However, in spite of their importance, the effects of
84 ENSO on the bacterial community is still unclear, despite the fact that these
85 microorganisms play central roles in the food web of the Patos Lagoon estuary (Abreu
86 *et al.* 1992) and in the macrophyte decomposition of this ecosystem (Anésio *et al.*
87 2003). Moreover, it is widely established that bacteria are strongly regulated by salinity
88 (Lozupone & Knight, 2007), which has recently been demonstrated for Patos Lagoon
89 estuary (They *et al*., submitted).
90 In this study we hypothesize that bacteria in Patos Lagoon estuary can also be affected
91 by ENSO. As a result, the well established systematic differences in bacterial
92 community composition (BCC) and metabolism associated to salinity gradients
93 generally observed in estuaries is also observed in Patos Lagoon estuary, but with a
94 strong temporal component associated to the ENSO phenomenon, i.e., freshwater

bacteria and their respective activity pattern dominate during and after an El Niño event (high precipitation, low salinity), while the opposite pattern is found in a situation of La Niña event (low precipitation, high salinity). In order to test this hypothesis, we assessed BCC through temporal temperature gradient electrophoresis (TTGE) and the bacterial community-level physiological profiles (CLPP, Ecoplate™ Biolog) at the mouth of the Patos Lagoon estuary, during one year encompassing the transition of a moderate El Niño to a strong La Niña event. Because precipitation is associated to higher nutrients availability (Odebrecht *et al*., 2005), we also investigated the role of resources (nutrients and substrates) and environmental conditions (temperature, salinity, pH and transparency) on the BCC and CLPP through Canonical Correspondence Analysis.

2. Material and Methods

2.1 Sampling and analyzed variables

The fixed sampling station is located close to the estuary mouth (32º 8’ 58.62” S / 52º 6’ 24” W) and inside the perimeter of the city of Rio Grande in southern Brazil (Figure 1).

Position of Figure 1

Samples were taken monthly between June 2010 and May 2011. The Southern Oscillation Index (SOI) data between January 2010 and December 2011 was acquired from the Australian Govern Bureau of Meteorology (2013, available at: http://www.bom.gov.au/climate/current/soihtm1.shtml) (Figure 2).

Salinity (Atago S/Mill-E refractometer), temperature (Labortherm mercury thermometer), and Secchi depth were measured in the field. Surface water was collected with a plastic bucket and an aliquot (5 L) was separated for chlorophyll *a* analysis, and one liter was pre-filtered through a plankton sieve (5.0 µm mesh size), in order to exclude most protozoans and metazoans, stored in autoclaved (121 °C, 30 minutes) borosilicate bottles, and kept in ice until filtration.

Chlorophyll *a* was determined after filtration of variables volumes through Whatman GF/F filters, extraction in the dark with cold (-12 °C) acetone (90% v/v), and fluorometric readings (Turner TD700 fluorometer) without acidification (Welschmeyer, 1994).

The pH (Digimed) was estimated in an aliquot of the pre-filtered water. Another aliquot (250 mL) was filtered through 0.2 µm, 47 mm Ø, polycarbonate filters for the concentration of bacteria (storage at -20 °C). In the filtrate, the relative concentration of low molecular weight substances was estimated at 250 nm (LMW) (UV-VIS CARY spectrophotometer) (Strome & Miller, 1978; Lindell *et al.*, 1995; Stepanauskas *et al.*, 2000). Another aliquot of the filtrate (250 mL) was stored in polyethylene flasks (-20 °C) for colorimetric analyses of NH_4^+ (UNESCO, 1983), NO_2^-, NO_3^- and PO_4^{-3} (Strickland & Parsons, 1972).

2. 2 Temporal Temperature Gradient Electrophoresis (TTGE)

The DNA from bacterial cells concentrated in filters was extracted with a commercial kit (QIamp DNA Stool, Qiagen), according to the manufacturer's recommendations (modification: cell lysis at 95 °C). The DNA quality was checked by 1% agarose gel electrophoresis. After extraction, around 180 bp within the V3 hypervariable region of the 16S bacterial gene was amplified through nested-PCR using the primers 11F (5'-

144 GTTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') (Siripong
145 & Rittman, 2007) in the first step and primers GC-338F (5'-CGC CCG CCG CGC GCG
146 GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG
147 - 3') and 518R (5'- ATT ACC GCG GCT GCT GG - 3') (Henriques *et al.*, 2006) in the
148 second step. The first step final concentrations were as follows: 1× Reaction Buffer
149 (initial 10×: 20 mM Tris-HCl (pH 8.0), 40 mM NaCl, 2 mM sodium phosphate, 0.1 mM
150 EDTA, 50 % v/v glycerol), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each
151 primer, 20 ng μL^{-1} of BSA, 0.2 µL of Platinum® Taq DNA polymerase (5 U μL^{-1},
152 Invitrogen) and 1.0 µL of template DNA in a 12.5 µL reaction. The PCR conditions
153 were: 5 minutes at 94°C, 30 cycles of 5 minutes at 94 °C, 1 minute at 52 °C and 1
154 minute 30 s at 72 °C. The last step was a final extension of 10 minutes at 72 °C.
155 The second step final concentrations were as follows: 1× amplification buffer (initial
156 10×: 20 mM Tris-HCl (pH 8.0), 12 mM $MgSO_4$, 40 mM KCl, 1 mM DTT, 0.1 mM
157 EDTA, 50% v/v glycerol), 0.3 mM of each dNTP, 0.3 µM of each primer, 1.0 µL of Pfx
158 50™ DNA polymerase (5 U μL^{-1}, Invitrogen) and 2.0 µL of template DNA from the
159 first step in a total volume of 50 µL. The PCR was conducted for 5 minutes at 94 °C,
160 followed by 20 touchdown cycles (van der Gucht *et al.*, 2007) with 1 minute at 94 °C, 1
161 minute starting at 65 °C and ending at 55 °C (decreasing 0.5 °C each cycle), and 1
162 minute at 68 °C. After the touchdown cycles, more 20 cycles were followed with 1
163 minute at 94 °C, 1 minute at 55 °C, 1 minute at 68 °C and 30 minutes at 68 °C of final
164 extension in order to minimize artefactual bands formation (Janse *et al.*, 2004). The
165 PCR products were checked for correct size in 1% agarose gel electrophoresis and
166 quantified with Quant-It™ dsDNA broad range assay kit (Invitrogen).
167 The TTGE was run in a DCode system (BioRad) with the following conditions
168 empirically tested: 14% polyacrylamide gels (37.5:1 acrylamyde:bis-acrylamyde), 7M

urea and 1.25× TAE buffer. The gel was polymerized with 50 µL of TEMED and 100 µL of APS 10% for 1.5-2 h and submitted to a pre-run at 200 V for approximately 30 minutes. Samples (~ 600 ng of PCR amplified DNA) were loaded in a proportion of 1:1 sample:gel loading dye (70% glycerol, 0.05% bromophenol blue, 0.05% xylenocyanol) and the run was set to 66.0-69.7 °C, 0.2 °C h^{-1} at 68 V (Lehours *et al.* 2010). The gel was stained in 1.25× TAE buffer containing 1 µg $µL^{-1}$ of ethidium bromide for 15 minutes and destained in 1.25× TAE buffer for the same time. The gel was photographed with UV transillumination with T1201 Sigma/Ultra Lum Ultra Cam Digital Imaging equipment coupled to a Power Shot A620 Canon Camera.

The calibration and alignment of band positions were based on Tourlomousis *et al.* (2010) with modifications. Instead of three standards, we used only an artificial standard made from a mixture of 6 bacterial isolates previously collected from the estuary. For this, approximately 100 ng of nested PCR amplified DNA (the same way as for the samples) was mixed together and the mix applied before and after the samples in the gel with the same procedure of the samples. Band detection and alignment were done with the help of the software Gel Analyzer 2010a (Lazar, 2010) after rolling ball background subtraction. Bands were detected automatically and then corrected by supervision. Band positions were corrected by reference values (Rf, a measurement of band position relative to its total length). The bands generated by the isolates were used to anchor smooth lines of same Rfs across gel images. This procedure allows for correction of gel distortion and within-gel correct alignments (Tourlomousis *et al*., 2010). The program Gel Analyzer generates matrices of discrete Rf values for each sample (not densitometric curves, like in Tourlomousis *et al*., 2010) and by default respective band raw volumes (peak area × peak height). Instead of using the software Matlab, band matching was carried in the software R 2.15.0 (R Development Core

194 Team, 2012) using values of Rf and raw volume through a cluster-based peak alignment
195 algorithm (Ishii *et al.*, 2009). Output matrices were converted to presence/absence
196 matrices and used in the subsequent statistical analyses.
197
198 *2.3 Community-Level Physiological Profiles (CLPP)*
199 The capacity of oxidation of 31 organic substrates (list of substrates in Figure 6) was
200 assayed in microtiter plates Ecoplate™ (BIOLOG). In the laboratory, the pre-filtered
201 water was inoculated (150 µL in each well) in the plates, which were incubated for 7
202 days at 20 °C in the dark. The time between sampling and start of the incubation was 2-
203 3 h. At the end of incubation, the wells were individually homogenized with automatic
204 pipette and read at 595 nm in a plate reader (TP Reader NM Thermoplate). The first
205 sampling month was read in a Bio-Tek ELx 800 plate reader at 630 nm. These readings
206 were corrected by an absorbance curve of 595 nm *versus* 630 nm. For this, the plate of
207 July was read by the two plate readers and a curve was constructed by regression (R^2 =
208 0.97; Abs595 = 1.1145[Abs630] - 0.0132) and applied to correct the June plate
209 readings.
210 The readings were subtracted from the reference well (redox dye only); negative values
211 were considered as zero. Corrected readings were divided by the AWCD (Average Well
212 Colour Development, the average of all corrected readings of each plate) in order to
213 normalize differences in initial bacterial density among samples (Garland & Mills,
214 1991).
215
216 *2.4 Nitrogen and phosphorus limitation assessment*
217 In order to assess a possible effect of nutrient limitation, two indexes were calculated
218 based on the consumption pattern of Ecoplate's™ nitrogen- and phosphorus-based

compounds. The Nuse index is a proportion calculated as the sum of the raw absorbances of the nitrogen-based compounds over the sum of absorbances of all compounds for a given plate, after correction for the control wells (Sala *et al*., 2006). This index is negatively correlated to the DIN concentration in environmental samples and increases in the index are reliable indicators of nitrogen limitation (Sala *et al*., 2006). Similarly, we also estimated a Puse index, based on the same principle, and using the phosphorus-based compounds. A summary of the nitrogen- and phosphorus-based compounds in Ecoplates™ can be found in Sala *et al.* (2006).

2.5 Data analyses

Correlation and cross-correlation of the pair SOI *versus* salinity was performed in the software PAST 2.14 (Hammer *et al.*, 2001) using monthly data encompassing 2 years (2010 and 2011). Differences between the means of environmental variables of a *posteriori* defined groups of samples were tested by t-test with permutation (10,000 permutations) using the package permute (Simpson, 2012) and the function pt.test (coded in: http://127.0.0.1:26109/library/permute/html/shuffle.html) in R 2.15.0 (R Development Core Team, 2012).

The temporal structure of the BCC and the CLPP was investigated through cluster analyses using UPGMA (Unweighted Pair Group Method with Arithmetic Mean). For I) BCC, we employed Bray-Curtis dissimilarity (presence/absence) after exclusion of bands with frequency <10%; and for II) CLPP we calculated correlation dissimilarity (1-correlation) based on AWCD transformed data (Hackett & Griffiths, 1997). The consistency of *a posteriori* groups of months was tested by Non-Parametric Multivariate Analysis of Variance (NPMANOVA) (Anderson, 2001) in software PAST 2.14 (Hammer *et al.*, 2001) (9,999 permutations), using the same similarity/dissimilarity

244 measures as in the clusters. The Bonferroni correction (n × p, where n is the number of
245 comparisons and p is the P-value obtained by permutation) was employed for *a*
246 *posteriori* contrasts in the PAST 2.14 software (Hammer *et al.*, 2001).
247 Canonical Correspondence Analyses (CCA) were run in order to identify the
248 explanatory variables (salinity, temperature, pH, secchi depth, pH, NH_4^+, NO_2^-, NO_3^-,
249 PO_4^{-3} and LMW substances) which significantly explained the variation in BCC and
250 CLPP (chlorophyll *a* was excluded because of missing data in January and May 2011).
251 For BCC, bands with frequency of occurrence <10% were excluded. The variables were
252 tested for variance inflation factors (VIFs) and variables with VIFs >10 were removed
253 according to the criterion of Borcard *et al.* (2011). The significance of global CCA and
254 of CCA axes was tested by permutation ($\alpha = 0.05$, 1000 iterations). The whole
255 procedure was based on Borcard *et al.* (2011) using R 2.15.0 (R Development Core
256 Team, 2012) and the packages Vegan (Oksanen *et al.*, 2011) and BiodiversityR (Kindt
257 & Coe, 2005).
258 All graphical displays were performed in R 2.15.0 (R Development Core Team, 2012)
259 and levels of significance for statistical tests were set to $\alpha = 0.05$.
260
261 **3. Results**
262
263 According to the Southern Oscillation Index (SOI), the sampling period encompassed
264 the transition of a moderate El Niño period that started in late 2009 and lasted until
265 March 2010 to a strong La Niña event (between July 2010 and April 2011) (Figure 2).
266 Two periods were distinguished in terms of environmental conditions. The first period,
267 between June and October 2010 (Period 1, P1) displayed low to intermediate salinity (<
268 20), whereas between November 2010 and May 2011 (Period 2, P2) the salinity

increased up to 36 (P1 × P2: t = -5.63, P-value < 0.01) (Figure 3A). In general, the salinity followed the behavior of the SOI with a time lag of 4 months (cross-correlation: r = 0.52; P-value = 0.019). The water temperature (P1 × P2: t = -3.12, P-value < 0.05), pH (P1 × P2: t = -3.86, P-value < 0.01, not shown) and Secchi depth (P1 × P2: t = -2.31, P-value = 0.056) were generally lower in P1, increasing after November 2011; the chlorophyll *a* showed the tendency to increase in P2, but the difference was not statistically significant (P1 × P2: t = -1.73, P-value = 0.138) (Figure 3B-D). The LMW substances decreased in P2, but not statistically significantly (P1 × P2: t = 1.16, P-value = 0.275) (Figure 3E).

Position of Figure 2

Position of Figure 3

The concentration of NO_3^- (P1 × P2: t = 3.48, P-value < 0.01) was higher in P1, with NO_2^- following the same tendency to decrease in P2, but not significantly (P1 × P2: t = 1.53, P-value = 0.136). NH_4^+ did not present consistent differences between periods (P1 × P2: t = -0.80, P-value = 0.429), with two peaks in August and October 2010 and a steady increase after November 2010 (Figure 4A-C). The Nuse index did not differ between the two periods (P1 × P2: t = 0.56, P-value = 0.591), but was generally higher from June to December 2010 and slightly lower afterwards (Figure 4D).
Similarly to nitrogen-based nutrients, PO_4^{-3} concentrations also decreased in P2 (marginally: t = 2.00, P-value = 0.076), but tended to increase after January 2011 (Figure 4E).The Puse index was very variable with a tendency of larger values after

December 2010 and the largest in February 2011, but did not differ between the two periods (t = -0.49, P-value = 0.647) (Figure 4F).

Position of Figure 4

The TTGE revealed a total of 44 bands, the frequency of occurrence of 38 being >10%. Of the total, 32% of band positions were exclusive to one of the periods, whereas the majority was present during the entire sampling period. When we analyze the band positions according to salinity ranges defined as limnetic-oligohaline (0-5), mesohaline (5-18) and polyhaline (18-40), about 36% were exclusive to each salinity range, 46% were shared between two out of three salinity ranges and 18% were common to all samples. Of the shared band positions, the same number was shared by limnetic-oligohaline and mesohaline samples and by polyhaline and mesohaline samples (Table 1; Figure 5).

Position of Table 1

A marked shift in BCC occurred between October and November 2010, originating two main clusters of samples separated at approximately 70% of dissimilarity: one cluster with samples from P1 and another cluster with samples from P2 (Figure 5). The difference between these two clusters was significant (NPMANOVA: *pseudo*-F = 7.44; P-value = 0.0015).

Position of Figure 5

318 The CLPP cluster also indicated a separation of two main clusters, at approximately
319 80% of dissimilarity: I) one cluster containing samples from July to November 2010
320 and also May 2011 (salinity 0-25), and II) another cluster containing samples from June
321 and December 2010, and January to April 2011 (salinity 15-36). The NPMANOVA
322 confirmed the consistency of this difference (*pseudo*-F: 5.73; P-value: 0.0023). Cluster I
323 was overall characterized by low substrate consumption (< 2 times the absorbance
324 divided by the AWCD) of most substrates, while the group cluster II showed the
325 utilization of smaller number of substrates, but some of them were consumed more
326 heavily (> 3 times the absorbance divided by the AWCD), like carbohydrates (α-D-
327 galactose), carboxylic acids (D-malic acid, α-ketobutyric acid, D-glucosaminic acid),
328 amino acids (L-serine, L-arginine), polymers (Tween 40, Tween 80, α-cyclodextrin),
329 and an ester (pyruvic acid methyl ester) (Figure 6).
330
331 ***Position of Figure 6***
332
333 The CCA of BCC was significant (*pseudo*-F = 1.68; P-value < 0.01), with 74.64% of
334 total variance accounted for by the explanatory variables (first axis: 27.20%; second
335 axis: 16.24% of the inertia explained). pH and NO_2^- had high VIFs and were excluded
336 from the analyses. All other variables had VIFs much smaller than 10 and remained
337 included. Only the first canonical axis was significant at α = 0.05 (CCA1: *pseudo*-F =
338 4.23; P-value = 0.022). The decreasing order of loadings in the first axis was as follows:
339 salinity, NO_3^-, temperature, Secchi depth, PO_4^{-3}, LMW and NH_4^+. The samples of P1
340 were associated to higher NO_3^-, PO_4^{-3} and LMW substances, whereas samples from P2
341 were associated to higher salinity, Secchi depth, temperature and NH_4^+ (Figure 7). The

342 CCA of CLPP was not significant (*pseudo*-F = 0.95; P-value > 0.50) and could not be
343 interpreted.
344
345 ***Position of Figure 7***
346
347 **4. Discussion**
348
349 Studies relating bacteria to ENSO are scarce. It has been found increases in abundance
350 of fecal coliforms when El Niño events coincide with winter (wet season) in Mississippi
351 Sound (Chigbu *et al*., 2005) and Charlotte Harbor, Florida (Lipp *et al*., 2001), with
352 impacts on the period open for shellfish harvesting (Chigbu *et al*., 2005). Also ENSO
353 has been found to influence positively the oxygenation of bottom waters of the
354 continental shelf of central Peru, favoring the development of microbial mats of the
355 sulphide-oxidizing *Thioploca* spp. (Gutiérrez *et al*., 2008) and negatively affect
356 bacterial production via primary negative effect on primary production along a 140º W
357 Equatorial Pacific transect (Kirchman *et al*., 1995). To our knowledge, this is the first
358 study at the community level that suggests a possible link between ENSO and bacteria.
359 We have found a succession from a low salinity (P1) to a high salinity (P2) period along
360 one year, associated to the transition of a moderate El Niño to a strong La Niña event.
361 The cascading effect of ENSO on hydrology has long been demonstrated for the region
362 of the Patos Lagoon estuary (Grim *et al*., 1998; Garcia *et al*., 2004; Abreu *et al*., 2010)
363 and prolonged drought associated to La Niña events (Grim *et al*., 1998) is the main
364 meteorological mechanism that promotes long time salinization of the estuary (Garcia *et*
365 *al*., 2004; Abreu *et al*., 2010), as observed in P2.

366 The change in salinity regime was associated to a marked shift in BCC and CLPP
367 between P1 and P2. The changes were sharpest and more consistent for BCC, but were
368 also significant for CLPP, as revealed by the NPMANOVA. These biological responses
369 were also associated to marked shifts in other environmental variables as increases in
370 temperature, pH, Secchi disk depth, chlorophyll *a* concentration, and decreases in NO_2^-,
371 NO_3^-, PO_4^{-3} and LMW substances. The BCC variability was significantly explained by
372 these environmental variables, as revealed by the CCA, which suggests that BCC can
373 be, to some extent, predicted by this group of variables.
374 The phylogenetic shift found can be explained by the general different bacterial
375 composition of fresh- and saltwater (Glöckner et al., 1999; Henriques et al., 2006;
376 Campbell & Kirchman, 2012). The exclusive band positions to each period (31.8%), or
377 salinity range (36.3%), indicated that an important fraction of the bacterial ribotypes
378 showed specificity to the salinity regimes. Most of the ribotypes, however, were found
379 across all samples and more than one salinity range, indicating that these bacteria may
380 be euryhaline/halotolerant. A few bacteria were only found in mesohaline conditions
381 (6.8%) and may be considered as exclusively estuarine bacteria. However, the TTGE
382 technique has the limitation of assessing only abundant bacteria (> 1-2% of frequency
383 of occurrence) in the PCR pool (Nocker *et al*., 2007), without any information on their
384 phylogenetic identity. Moreover, it may include inactive and dead bacteria that are not
385 contributing to the profile of the actually growing bacteria. Thus, partial genomic
386 sequencing of these specific and widespread ribotypes is needed for their taxonomic
387 identification and more detailed characterization of the bacterial taxonomic composition
388 in each salinity range.
389 The CCA did not significantly explain the variance of CLPP, which suggests that
390 bacterial physiological activity was not consistently constrained by environmental

391 variables as BCC. One possible explanation is that the plasticity of bacterial metabolic
392 responses adds noise to the physiological responses. Leflaive *et al*. (2008) demonstrated
393 that even similar bacterial communities under different phosphorus and nitrogen
394 conditions show variable CLPP responses. Moreover, shifts in CLPP can be very fast,
395 thus capable of following salinity changes in a tidal cycle (Thottathil *et al*., 2008).
396 The shifts in patterns of organic substrates consumption with decrease in richness of
397 substrates consumed and increase in the consumption of amino acids and carboxilic
398 acids in higher salinity waters observed in this study has been reported for an Indian
399 estuary (Thottathil *et al*., 2008) and recently for the Patos Lagoon estuary (They *et al*.,
400 submitted). Other studies employing Biolog plates (Schultz & Ducklow, 2000) and
401 culture independent methods (del Giorgio & Bouvier, 2002; Trousselier *et al*., 2002)
402 have also demonstrated consistent changes in bacterial activity along salinity gradients,
403 which suggests that these changes may be a common pattern of estuaries. Moreover,
404 December 2010 was marked by an increase in Nuse index, suggesting that bacteria
405 experienced an acute episode of nitrogen limitation, as also indicated by the decrease in
406 NO_2^- and NO_3^- in October 2010. All these information indicate that especially saltwater
407 bacteria can play a major role in the decomposition and mineralization of organic matter
408 abundantly found in estuaries, since these microorganisms generally come from
409 conditions of nutrients scarcity, probably having a large repertoire of enzymes ready to
410 act when these bacteria enter the estuary.
411 Increase in NH_4^+ concentration during drought events following La Niña have been
412 previously recorded in the Patos Lagoon estuary, which has been attributed to
413 macrophyte decomposition, or increases in sewage influence due to the longer water
414 residence time (Niencheski & Windom, 1994; Baumgarten *et al*., 1995; Abreu *et al*.,
415 2010). However, other possible explanation could be that saltwater bacteria are very

416 efficient in decompose and mineralize nitrogen from the organic matter. Since the
417 steady increase in NH_4^+ was not followed by increases in NO_2^- and NO_3^-, failures in
418 nitrification could have occurred leading to the ammonium accumulation.
419 There are indications that nitrification rates are higher at intermediate salinity
420 (Magalhães *et al*., 2005), being even inhibited at high salinity (Rysgaard *et al.*, 1999;
421 Magalhães *et al*., 2005), probably due to the lower diversity of ammonia oxidizing
422 bacteria with increasing salinity (Bernhard *et al*., 2005) or salinity stress of these
423 bacteria (Rysgaard *et al.*, 1999). The marginally significant cross-correlation between
424 NH_4^+ and NO_2^- at a 2 month time lag (r = 0.62; P-value = 0.054), reinforce the
425 hypothesis that the nitrification process might have occurred during the freshwater
426 period, but ceased/decreased with salinity increase, leading to the ammonium
427 accumulation during the drought period, in addition to increased mineralization.
428 There was no significant correlation between the Puse index and the PO_4^{-3}
429 concentration, which could be due to the rapid cycling of this element in this estuary
430 and also release of phosphate from the sediment after the input of saltwater (Abreu et
431 al., 1995; Windom *et al.*, 1999). The mineralization of PO_4^{-3} can be a dominant process
432 responsible for the higher phosphate concentration in the water during specific events,
433 which is in agreement with previous findings that primary production in the upper part
434 of Patos Lagoon sometimes depletes the inorganic nutrients (Odebrecht *et al*., 2010),
435 whereas in the low estuary the primary production is supported by mineralization of
436 organic phosphorus and nitrogen (Niencheski & Windom, 1994; Windom *et al*., 1999;
437 Odebrecht *et al*., 2005). In the period of low phosphate (second period), a peak in Puse
438 may be due to the response of saltwater bacteria to phosphorus limitation by increase in
439 phosphatase activity (Hoppe, 2003), enhancing its mineralization.

Interactions between meteorology and hydrology caused by ENSO directly affect the dynamics of plankton, benthic organisms, and fish in the Patos Lagoon estuary often with economic repercussions on the regional fish and shrimp fisheries (Odebrecht *et al.*, 2010). From an ecological point of view, the phytoplankon is the closest group to bacteria in Patos Lagoon estuary and a clear influence of ENSO on the microalgae has been demonstrated for this estuary, with profound changes of phytoplankton composition and production in El Niño and La Niña periods. For instance, it has been found that phytoplankton responds to nutrients that are changed by hydrology that at its turn is altered by El Niño events by increasing the levels of production and biomass accumulation, with direct influence on the estuarine food web. On the other hand, phytoplankton presents low biomass mainly due to nutrients scarcity during La Niña (Abreu *et al*., 2010; Odebrecht *et al*., 2010).

Besides the effect of salinity, our results showed changes in bacterial diversity and activity associated to different environmental conditions. During the study period these changes were coincidental with a transition from a moderate El Niño to a strong La Niña event, thus suggesting a link between bacteria and ENSO. Because bacteria have a prominent role on decomposition and mineralization, the higher consumption of organic nitrogen and phosphorus found in the second period suggests that these elements could undergo higher rates of mineralization during prolonged drought periods and high salinity, characteristic of La Niña periods. Thus, bacteria would respond to nutrient limitation, possibly increasing rates of mineralization of organic nitrogen and phosphorus and decreasing rates of nitrification, mainly affecting the nitrogen cycles during La Niña periods.

In summary, the results of this study highlight possible important biogeochemical implications for Patos Lagoon estuary in response to meteorological and hydrological changes related to the ENSO phenomena. However, it is important to stress that further detailed studies are needed to evaluate this relationship between ENSO episodes and the bacterial composition and activity in the Patos Lagoon estuary. Moreover, other aspects need to be deeper investigated as well through experimental and field approaches, in order to evaluate i) the degree of functional redundancy between low and high salinity bacterial communities and their phylogenetic identity; ii) the follow-up of rates of consumption of substrates by culture-independent approaches and other ecological functions like decomposition processes; iii) changes in microbial food web functioning and interaction between bacteria and primary producers.

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687 **Figure legends**

688

689 **Fig. 4.** Sampling area: Patos Lagoon estuary (southern Brazil), with sampling station
690 indicated by the black dot. The closest city to the sampling point (Rio Grande city) is
691 also shown on the map.

692

693 **Fig. 2.** South Oscillation Index (SOI) between January 2010 and December 2011.
694 Months are indicated by their first letter. Indexes < -8 indicate El Niño, whereas indexes
695 > +8 indicate La Niña events; these thresholds are represented as grey pointed lines.

696

697 **Fig. 3.** Main environmental variables at the mouth of the Patos Lagoon estuary between
698 June 2010 and May 2011. Months are indicated by their first letter, beginning by June
699 2010.

700

701 **Fig. 4.** Dissolved inorganic nutrients and indicators of nitrogen (Nuse) and phosphorus
702 (Puse) limitation at the mouth of the Patos Lagoon estuary between June 2010 and May
703 2011. Months are indicated by their first letter, beginning by June 2010.

704

705 **Fig. 5.** Cluster analysis of bacterial community composition (BCC) in the mouth of
706 Patos Lagoon estuary between June 2010 and May 2011 (the salinity of the samples is
707 indicated inside parentheses). Exclusive band positions (> 10% frequency) per period
708 are indicated by arrows and numbers: bottom (low salinity: between June and October
709 2010) and top (high salinity: between November 2010 and May 2011). A few bands
710 could not be visually identified in the image and were not indicated.

711

712 **Fig. 6.** Cluster analysis of Community-Level Physiological Profiles (CLPP) in the
713 mouth of Patos Lagoon estuary between June 2010 and May 2011. The degree of
714 consumption of substrates is arbitrarily scaled: Xi corresponds to the corrected
715 absorbance at 595 nm (raw absorbance minus control well absorbance) of each substrate
716 divided by the plate's respective AWCD. Classes of substrates: (□) carbohydrate, (■)
717 carboxylic acid, (Δ) amino acid, (▲) polimer, (○) phosphorilated chemical, (●) amine,
718 (♦) ester.
719
720 **Fig. 7.** Canonical Correspondence Analysis (CCA) of bacterial community composition
721 (BCC) in the mouth of Patos Lagoon estuary between June 2010 and May 2011. Sample
722 labels identified by months: Jan, January; Feb, February; Mar, March; Jun, June; Jul,
723 july; Aug, August; Sep, September; Oct, October; Nov, November; Dec, December.
724 Environmental variables: Sal, salinity; Temp, temperature; Secchi, secchi depth; NH_4^+,
725 ammonium ion; NO_3^-, nitrate; PO_4^{-3}, ortophosphate; LMW, low molecular weight
726 substances.
727
728
729
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735
736

737 **Table**

738

739 **Table 1.** Total and proportion of exclusive, exclusive shared and shared ribotypes (band
740 positions) for the two periods and by salinity ranges detected by TTGE in Patos Lagoon
741 estuary between June 2010 and May 2011. First period (P1): between June and October
742 2010; Second period (P2): between November 2010 and May 2011.

Band positions	Period	Nº bands	%
Exclusive	First (Low Salinity)	8	18.2
	Second (High Salinity)	6	13.6
Shared	All	30	68.2
Total		44	100.0

Band positions	Salinity range	Nº bands	%
Exclusive	L-O	6	13.6
	M	3	6.8
	P-M	7	15.9
Shared	L-O × M	9	20.5
	L-O × P-M	2	4.5
	M × P-M	9	20.5
	All	8	18.2
Total		44	100.0

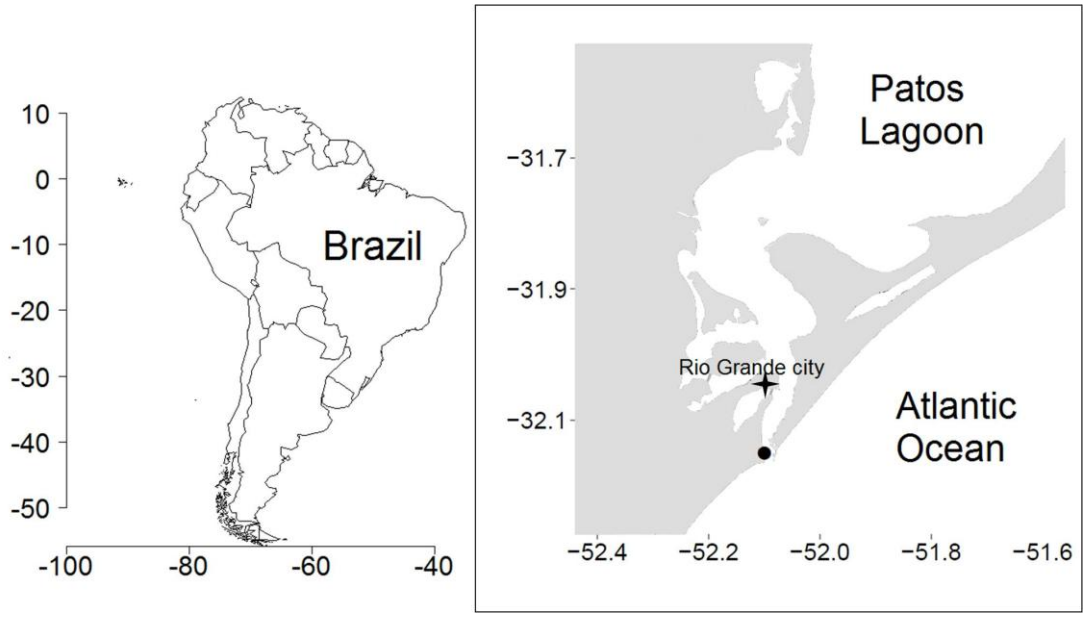
743 Salinity ranges: Limnetic-Oligohaline (L-O, 0-5); Mesohaline (M, 5-18); Polyhaline-
744 Mixohaline (P-O, 18-40).

745

746

747 **Figures**

748



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750 Figure 1

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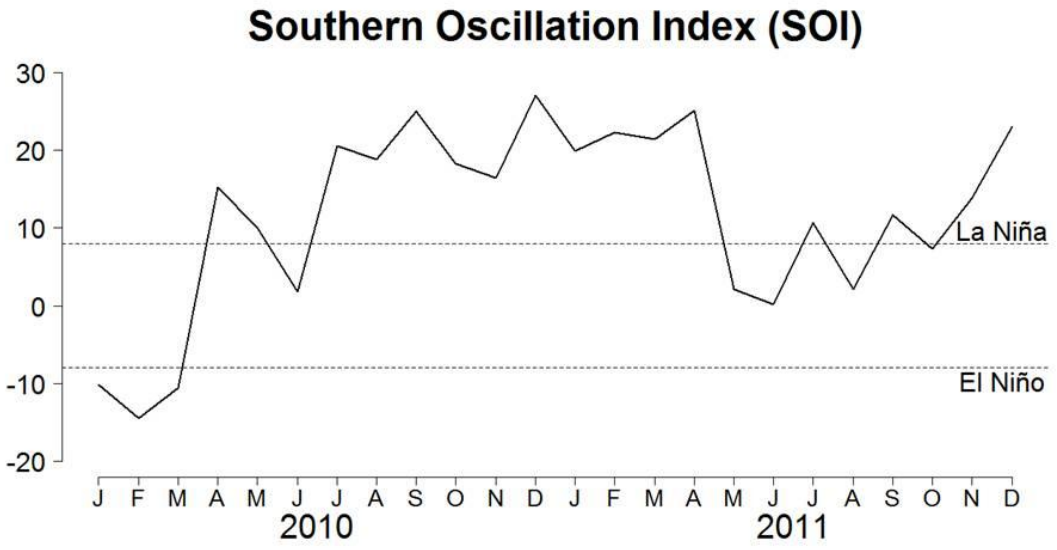

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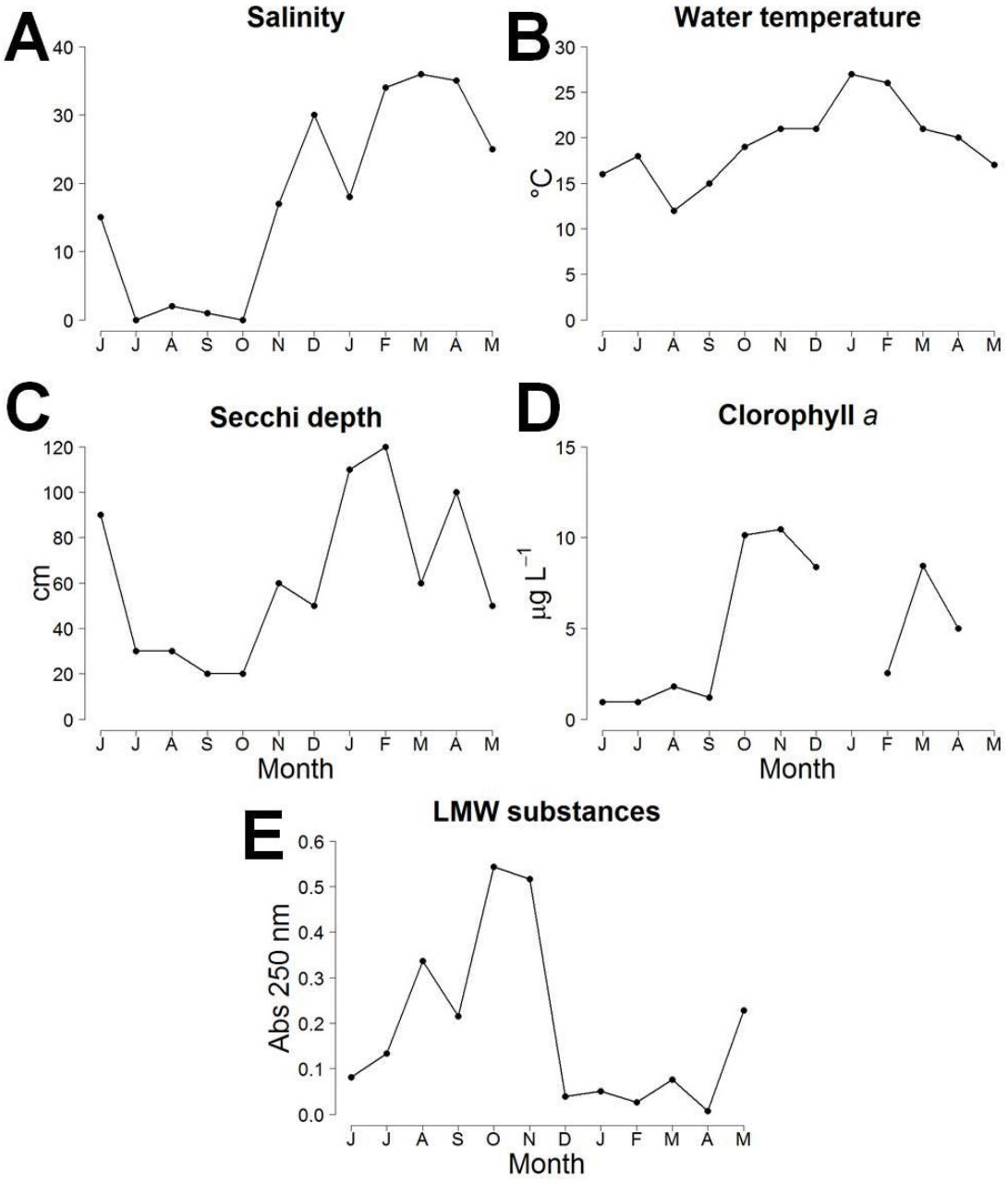
753 Figure 2



754

755 Figure 3

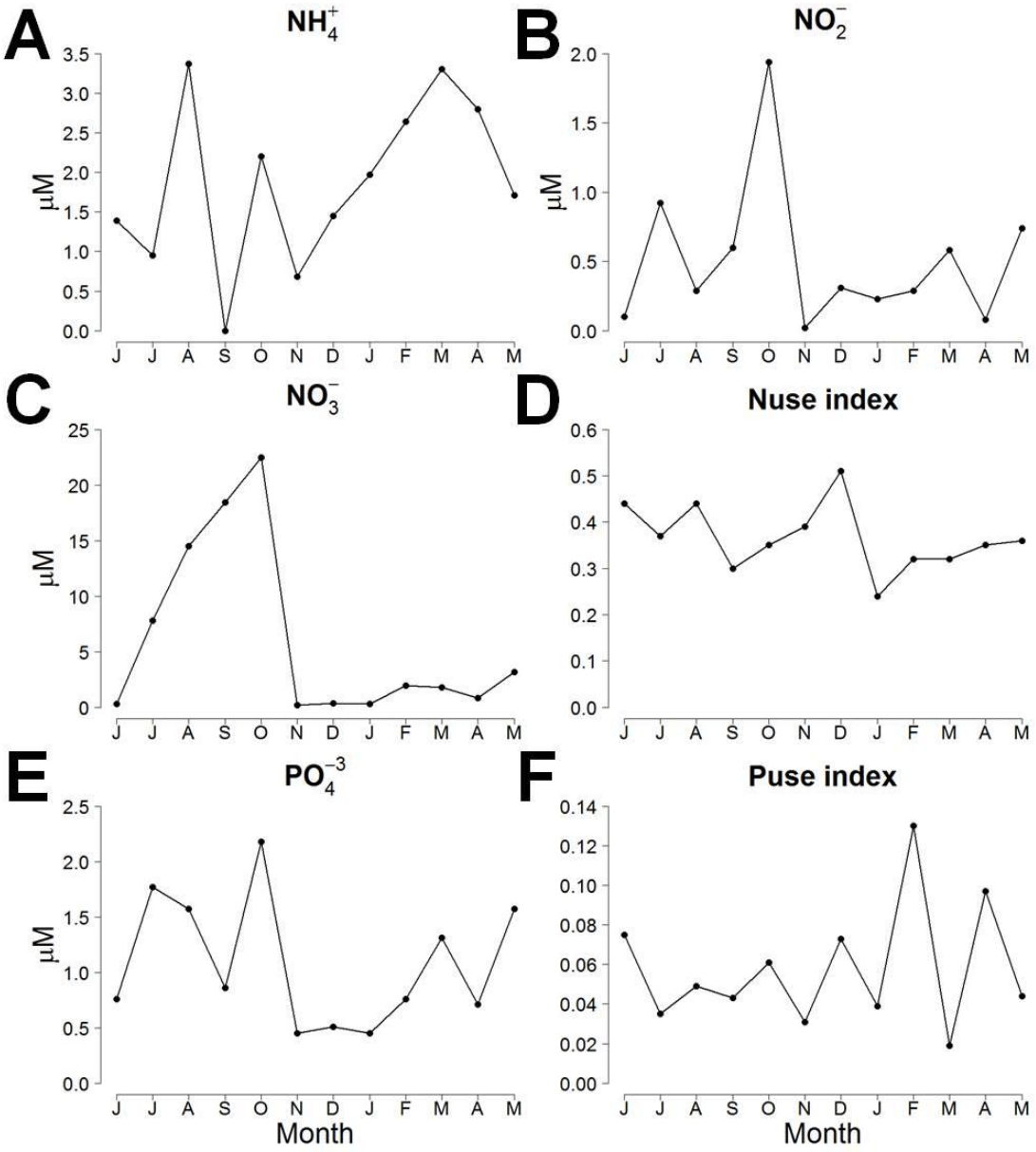
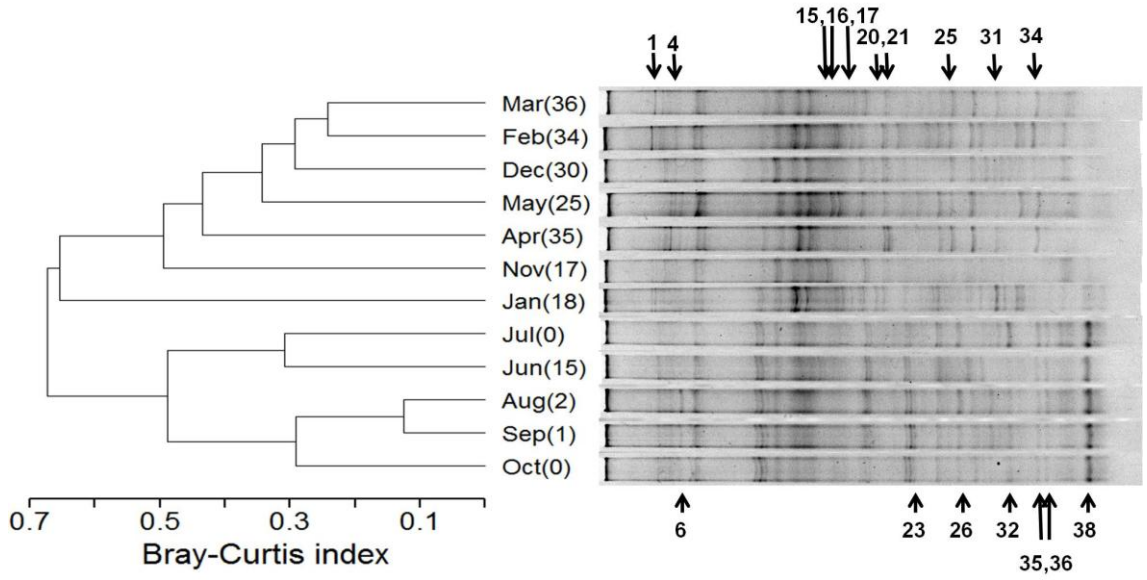

A
NH_4^+
B
NO_2^-
C
NO_3^-
D
Nuse index
E
PO_4^{-3}
F
Puse index
µM
J J A S O N D J F M A M
Month

Figure 4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Mar(36)
Feb(34)
Dec(30)
May(25)
Apr(35)
Nov(17)
Jan(18)
Jul(0)
Jun(15)
Aug(2)
Sep(1)
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Bray-Curtis index
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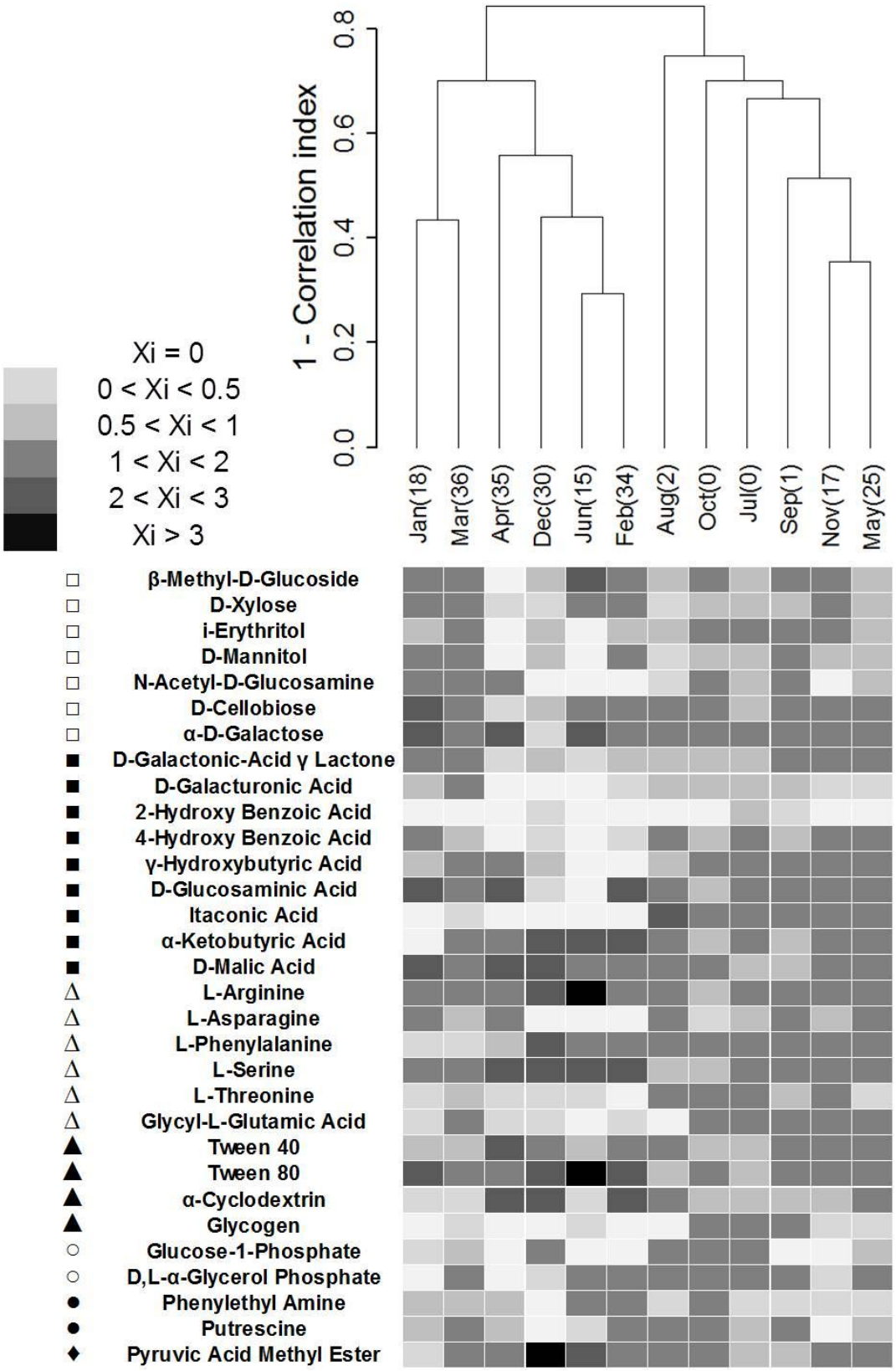
Figure 5



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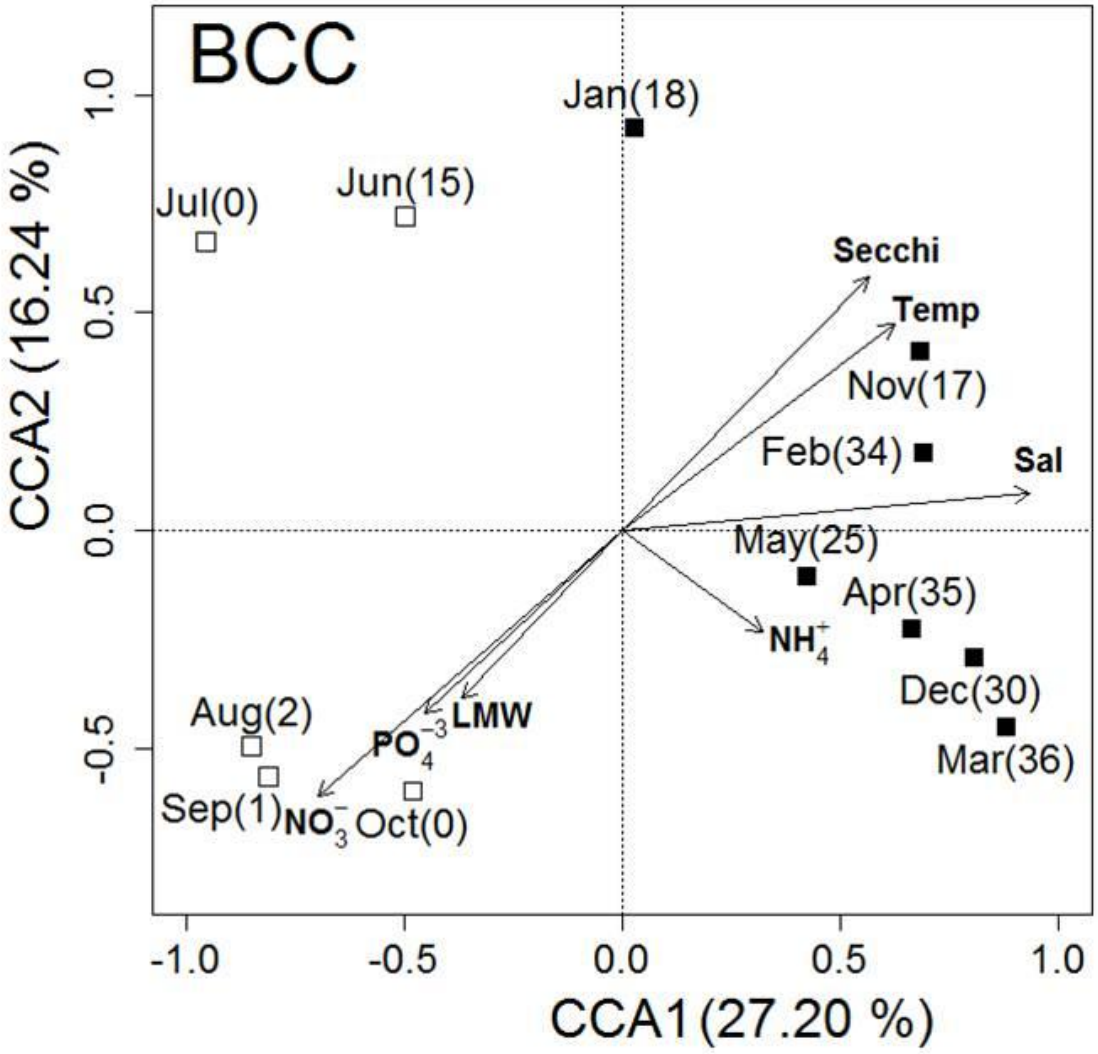
761 Figure 6



762

763 Figure 7

APÊNDICE 3

Apresentação

Este apêndice refere-se ao artigo intitulado: “Distinct responses of bacterioplankton community composition and activity to salinity and nutrients gradients along the microtidal Patos Lagoon estuary”, dos autores Ng Haig They, Lise Maria Holanda Ferreira, Luís Fernando Marins, Osmar Olinto Möller Jr. e Paulo Cesar Abreu.

Este artigo está em preparação para submissão na revista “Estuarine Coastal and Shelf Science”.

Distinct responses of bacterioplankton community composition and activity to salinity and nutrients gradients along the microtidal Patos Lagoon estuary

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Running title: Salinity and nutrients effect on bacterial composition and activity

Key-words: Temporal Temperature Gradient Gel Electrophoresis / ECOPLATE™ / bacteria

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Abstract

Most studies focusing bacterioplankton variability have been conducted in tide-dominated estuaries, whereas microtidal systems have been highly overlooked. In this study the spatial (longitudinal and depth) and short-term temporal variation (~1 day) of bacterial community composition (BCC) and activity were evaluated in the microtidal and dynamic Patos Lagoon estuary (southern Brazil). Samples were taken at the surface (May 23 2012) and at surface and bottom (May 24 2012) at a transect along the main estuarine longitudinal axis (~74 km long) of the estuary. The BCC was assessed through temporal temperature gradient gel electrophoresis (TTGE) and the activity through community-level physiological profiles (CLPP, Ecoplate™ - Biolog), along with salinity and physical/chemical variables measurements. The spatial and temporal patterns were investigated through cluster analyses and the effect of salinity and environment variables was assessed through Canonical Correspondence Analysis (CCA). A salt wedge intrusion on the second day caused a marked increase in salinity, nutrients and seston loads. In response, significant variations of BCC and CLPP occurred between days, between surface and bottom samples and stations. Salinity and environmental variables were the main forcing functions on the structuring of BCC and CLPP, but the unexpected higher nutrient content and bacterial activity in saltwater suggested that bacteria activity responded primarily to bottom-up effects like nutrient availability, whereas salinity is the main factor structuring BCC in this estuary. Thus, the accepted pattern of decreasing bacterial activity towards saltwater must be reconsidered, especially in estuaries where high concentrations of nutrients are eventually associated to high salinity waters.

1. Introduction

Spatial patterns of bacterial community composition and activity in estuaries considering longitudinal (Crump *et* al., 2004; Kirchman *et* al., 2005; Herlemann *et al*., 2011) and depth changes (Ribeiro *et al*., 2011) revealed systematic variations in bacterial community composition (BCC) along salinity gradients, with the dominance of β-, δ-, ε-Proteobacteria and Actinobacteria in freshwater and of α-Proteobacteria and γ-Proteobacteria in saltwater (Glöckner *et al*., 1999; Crump *et al*., 2004; Campbell and Kirchman, 2012).

The bacterial activity also seems to be strongly regulated by salinity in estuaries, decreasing towards higher salinity waters, as measured by independent methods like thymidine and leucine incorporation (Trousselier *et al*., 2002; del Giorgio and Bouvier, 2002), percentage of actively respiring (CTC+), membrane intact and high DNA bacteria (del Giorgio and Bouvier, 2002) and ectoenzymatic activity (Cunha *et al*., 2000).

However, the bulk of the studies that have dealt with bacterioplankton variability were focused in tide-dominated estuaries, whereas microtidal estuaries have been highly overlooked. Microtidal estuaries do not display the cyclic variations associated to tides, but may present large shifts in environmental conditions in short-scales (hours to ~ days) associated to meteorological forcing functions like winds, rainfall and evaporation (Möller *et al*., 2001), waves (Perillo, 1995) and river discharge (Luettich Jr. *et al.*, 2002). This variation can act on several time- and spatial scales with, sometimes, a clear seasonal variability (Hartmann and Schettini, 1991) or even showing the influence of global-scale climatic phenomena like El Niño Southern Oscillation - ENSO (Garcia *et al*., 2004; Abreu *et al*., 2010).

The Patos Lagoon estuary is a microtidal system where bacterial community composition (BCC) and the activity (through community-level physiological profiles – CLPP) have been recently investigated (They *et al*., in press). However, these studies were carried out in large temporal scales (monthly sampling program) and small spatial scale (one, or few sampling stations) (They *et al*., unpublished results).

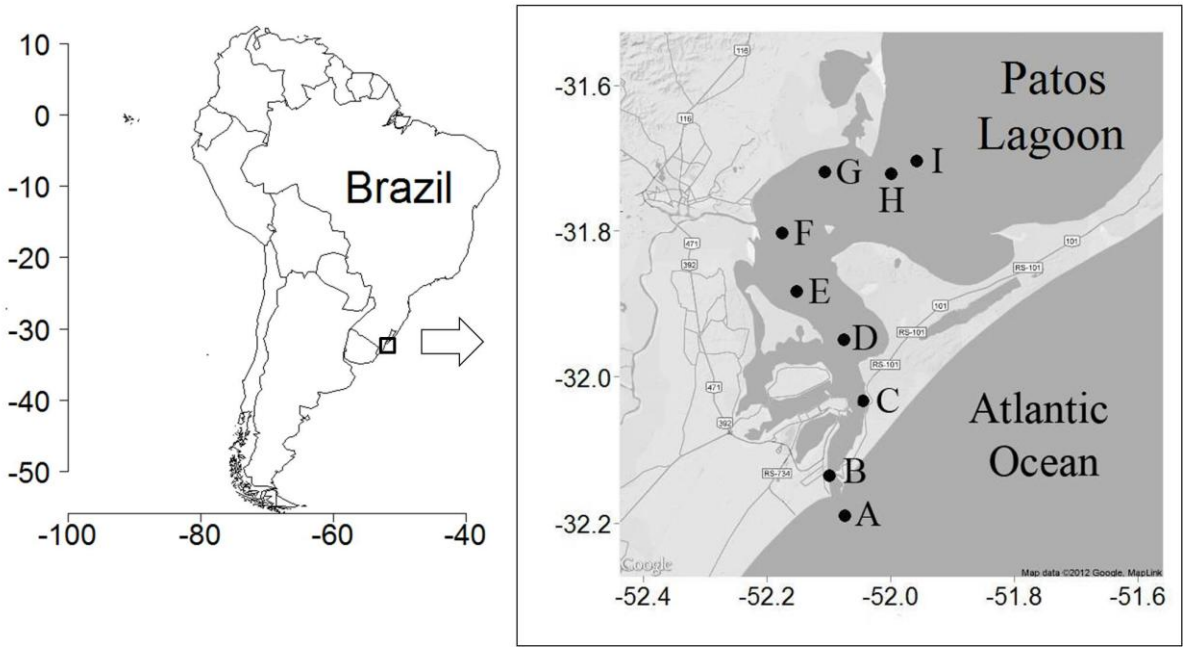
In this study the BCC and CLPP were evaluated in a short time scale (days), though covering the main longitudinal axis of the Patos lagoon estuary (ca. 74 km long). The results showed similar patterns of bacterial composition associated to salinity as identified in the previous studies, but the bacterial activity displayed an unexpectedly response with increasing activity towards nutrient rich saltwater.

2. Material and Methods

2.1 Study area and sampling design

The Patos Lagoon, located in south Brazil, is the largest choked coastal lagoon in the world (Kjerfve, 1986), connecting to the Atlantic Ocean through a narrow channel of 20 km length and 0.5-3 km wide (Asmus, 1997). It has 10,360 km^2 and is located inside a 200,000 km^2 watershed (Kjerfve, 1986), presenting a mean river discharge of 2,000 m^3 s^{-1} (Monteiro *et al*., 2011). The estuarine region comprises approximately 10% of the Lagoon's surface and presents a microtidal regime (± 0.4 m) due to the proximity of an amphidromic point (Soares and Möller Jr., 2001). The salinity is controlled mainly by wind, precipitation and evaporation. Because the lagoon extends in the northeast/southwest direction, southwesterly winds (autumn and winter) and low precipitation (summer) promote saltwater intrusion, whereas northeasterly winds (spring and summer) and high precipitation (end of winter and beginning of spring) intensify

101 the river discharge (Hartmann and Schettini, 1991; Marques *et al*., 2009; Möller *et al*.,
102 2001; Möller and Fernandes, 2010). However, the estuary is very dynamic and at any
103 time and location the salinity may shift drastically within a period of a few hours
104 (Möller and Fernandes, 2010). The mixing zone can achieve the inner continental shelf
105 during high river discharge and during drought periods the salt penetration can reach the
106 central part of the lagoon (~150 km from the lagoon's mouth) (Möller *et al.*, 2001), but
107 generally it is close to the station I (Fig. 1).
108 The nine sampling stations were distributed along the main longitudinal axis (ca. 74
109 km), of the estuary (Fig. 1). Samples were taken at days May 23rd 2012 and May 24th
110 2012 at the surface in the first day (direction: A to H) and at the surface and bottom in
111 the second day in the opposite direction (I to B) (Fig. 1; Table 1). Surface samples were
112 collected with a plastic bucket and bottom samples were taken with a 5 L Niskin bottle,
113 1 m above the sediment (Table 1).
114



115

116 **Fig. 1:** Sampling area: Patos Lagoon estuary (southern Brazil), with sampling stations
117 indicated by black dots and capital letters (A-I). The estuarine region extends up to the
118 point I under regular hydrological conditions.

Table 1 Transect of sampling stations (A-I) collected at the Patos Lagoon estuary during two days. Samples were collected at the surface (S) and at the bottom (B, 1 m above the sediment).

	Sampling depth (m)																	
	A		**B**		**C**		**D**		**E**		**F**		**G**		**H**		**I**	
	S	**B**	**S**	**B**	**S**	**B**	**S**	**B**	**S**	**B**	**S**	**B**	**S**	**B**	**S**	**B**	**S**	**B**
23/05/2012	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	-	-
24/05/2012	-	-	0	15	-	5	0	11	0	5	-	5	-	6	0	-	0	-
Distance from estuary mouth (km)	0		6.6		19.1		28.8		39.1		48.3		59.7		69.8		74.1	

(-), samples not taken.

122 *2.2 Environmental variables*

123 In each station, the vertical profile of temperature was measured *in situ* with an YSI 30
124 30-50-FT probe and salinity was measured in the collected water with an Atago S/Mill-
125 E refractometer. An aliquot of the water was immediately filtered onto Whatman GF/F
126 glass fiber filters for the determination of chlorophyll *a* and seston and filters were
127 frozen (-20 ºC) until analysis. The fluorescence of chlorophyll *a* extracted with acetone
128 (90% v/v) for 24 h (-12 ºC) in the dark was read by calibrated fluorometry (Turner
129 TD700 fluorometer) without acidification (Welschmeyer, 1994). Seston was measured
130 gravimetrically (Lenz, 1972).

131 One liter of water was pre-filtered through a plankton sieve (5.0 µm mesh size) for the
132 exclusion of protozoans and metazoans. The pre-filtered water was filtered (250 mL)
133 onto 0.2 µm, 47 mm Ø polycarbonate filters for the bacterial DNA extraction and
134 immediately stored at -20 °C. One aliquot of the filtered water was frozen in
135 polyethylene flasks at -20°C for the determination of dissolved inorganic nutrients NH_4^+
136 (Unesco, 1983), NO_2^-, NO_3^- and PO_4^{-3} (Strickland and Parsons, 1972). Another aliquot
137 of the filtered water was frozen (-20 ºC) in muffled (450ºC, > 3h) amber glass vials for
138 the gross estimate of low molecular weight (LMW) substances through the
139 spectrophotometric absorbance at 250 nm (UV-VIS CARY spectrophotometer) (Strome
140 and Miller, 1978; Lindell *et al.*, 1995; Stepanauskas *et al.*, 2000).

141 The remaining pre-filtered water was stored in clean glass bottles (autoclaved 30
142 minutes at 121 ºC) and kept at room temperature until the end of the cruise. In the
143 laboratory, the pH was estimated in an aliquot of the pre-filtered water with a Digimed
144 potentiometer.

145

146

2.3 BCC - Temporal Temperature Gradient Gel Electrophoresis (TTGE)

The bacterial DNA was extracted from polycarbonate filters with the commercial kit QIamp DNA Stool (Qiagen), with cell lysis at 95 °C. The DNA quality was checked by 1% agarose gel electrophoresis.

Amplicons of approximately 180 bp were generated by nested-PCR employing the primers 11F (5'-GTTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') (Siripong and Rittman, 2007) and GC-338F (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG - 3') and 518R (5'- ATT ACC GCG GCT GCT GG - 3') (Henriques *et al.*, 2006). The region amplified comprises the V3 hypervariable region inside the bacterial 16S gene.

The final concentrations of the PCR reactions of the first step were as follows: 1× Reaction Buffer (initial 10×: 20 mM Tris-HCl pH 8.0, 40 mM NaCl, 2 mM sodium phosphate, 0.1 mM EDTA, 50 % v/v glycerol), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer, 20 ng μL^{-1} of BSA, 0.1 µL of Platinum® Taq DNA polymerase (5 U μL^{-1}, Invitrogen) and 1.0 µL of template DNA in a 12.5 µL reaction. The PCR conditions were: 5 minutes at 94°C, followed by 30 cycles of 5 minutes at 94 °C, 1 minute at 52 °C and 1 minute and 30 s at 72 °C. The final extension was of 10 minutes at 72 °C.

The final concentrations of the PCR reactions of the second step were as follows: 1× amplification buffer (initial 10×: 20 mM Tris-HCl pH 8.0, 12 mM $MgSO_4$, 40 mM KCl, 1 mM DTT, 0.1 mM EDTA, 50% v/v glicerol), 0.3 mM of each dNTP, 0.3 µM of each primer, 1.0 µL of Pfx 50™ DNA polymerase (5 U μL^{-1}, Invitrogen) and 2.0 µL of the first step DNA amplicons in a total volume of 50 µL. The PCR conditions were: 5 minutes at 94 °C, followed by 20 touchdown cycles (van der Gucht *et al.*, 2007) with 1

minute at 94 °C, 1 minute starting at 65 °C and ending at 55 °C (decreasing 0.5 °C each cycle) and 1 minute at 68 °C. Additional 20 cycles followed with 1 minute at 94 °C, 1 minute at 55 °C and 1 minute at 68 °C. The final extension was of 30 minutes at 68 °C in order to minimize the formation of artifactual bands (Janse *et al.*, 2004). The PCR products were checked for correct size through 1% agarose gel electrophoresis and quantified with Quant-It™ dsDNA broad range assay kit (Invitrogen).

The amplicons were electrophoresed in the DCode system (BioRad) based on the recommendations of the manufacturer with several modifications empirically tested: 14% polyacrylamide gels (37.5:1 acrylamyde:bis-acrylamyde), 7M urea and 1.25× TAE buffer. Gels were polymerized with 50 µL of TEMED and 100 µL of APS 10% for 1.5-2 h and submitted to a pre-run at 200 V for approximately 30 minutes. Samples (~ 600 ng of PCR amplified DNA) were loaded in a proportion of 1:1 sample:gel loading dye (70% glycerol, 0.05% bromophenol blue, 0.05% xylenocyanol). The run was set to 66.0-69.7 °C, 0.2 °C h^{-1} at 68 V (Lehours *et al.* 2010). Gels were stained in 1.25× TAE buffer containing 1 µg μL^{-1} of ethidium bromide for 15 minutes and distained in 1.25× TAE buffer for the same time. The gels were photographed with ultraviolet transillumination via T1201 Sigma/Ultra Lum Ultra Cam Digital Imaging equipment coupled to a Power Shot A620 Canon Camera.

The gels images were submitted to a procedure that allows for correction of between and within gel distortion and allows for correct alignments of band positions. This procedure was based on Tourlomousis *et al*. (2010) after modifications. From 6 bacterial isolates obtained previously from the Patos Lagoon estuary and submitted to the same procedure of PCR amplification of the samples, we constructed a "clone standard"; after amplification, 100 ng of the amplified DNA of each isolate was mixed together and applied before and after the samples in each gel. The band positions were

197 detected automatically after rolling ball background subtraction of the images with the
198 software Gel Analyzer 2010a (Lazar, 2010) and inspected afterwards for necessary
199 correction. The positions of bands in the "clone standard" were used to anchor smooth
200 lines that represented the same reference values (Rf, a measurement of the band position
201 relative to the lane length) across the image(s). After this alignment, we generated
202 matrices of the corrected band positions (not densitometric curves, like in Tourlomousis
203 *et al*., 2010) and respective band raw volumes (peak area × peak height). Even though
204 we did not use raw volumes, they are necessary for the algorithm employed for band
205 matching in the next step and are generated by default by Gel Analyzer. In order to
206 determine the band matching across lanes (same band positions after given cut-off),
207 these output matrices were subjected to cluster-based peak alignment algorithm (Ishii *et*
208 *al.*, 2009) in software R 2.15.0 (R Development Core Team, 2013) using a cut-off of
209 0.01. The resultant matrices of matched band positions were converted to
210 presence/absence matrices and used in the subsequent statistical analyses.
211
212 *2.4 Bacterial activity - Community-Level Physiological Profiles (CLPP)*
213 This assay was carried out in microtiter plates Ecoplate™ (Biolog), which indicate the
214 capacity of degradation of 31 organic substrates through the redox indicator tetrazolium
215 violet. Of the pre-filtered water, 150 µL was inoculated in each well and the plate was
216 incubated for seven days at 20 °C in the dark. The plates were read at 595 nm in a plate
217 reader (TP Reader NM Thermoplate). The time between sampling and incubation was
218 of approximately 35 h for the first sample (station A, 23/05/12), with decreasing times
219 for subsequent samples.

220 The readings were subtracted from the reference well (redox dye only), being
221 considered as zero the negative values. Raw readings were divided by the AWCD
222 (Average Well Colour Development) (Garland and Mills, 1991).
223 *2.5 Nuse and Puse indexes*
224 Two indexes that indicate nitrogen (Nuse) and phosphorus (Puse) limitation were
225 calculated based on the consumption of nitrogen- and phosphorus-based compounds.
226 The Nuse index was calculated as the sum of the absorbances of the nitrogen-based
227 compounds over the total absorbances of all compounds for a given plate, after
228 correction for the control wells (Sala *et al*., 2006). This index has been experimentally
229 proved to be a reliable indicator of nitrogen limitation, being negatively correlated to the
230 DIN (dissolved inorganic nitrogen) concentration in environmental samples (Sala *et al*.,
231 2006). Similarly, the Puse index was calculated in the same manner and taking into
232 account the phosphorus-based compounds. A complete list of the nitrogen- and
233 phosphorus-based compounds of Ecoplate™ can be found in Sala *et al.* (2006).
234
235 *2.6 Statistical data analyses*
236 The spatial and temporal patterns in BCC and CLPP were inspected through cluster
237 analyses (unweighted pair group method with arithmetic mean). For BCC we used the
238 Bray-Curtis dissimilarity index on the presence/absence matrices after exclusion of
239 ribotypes with frequency of occurrence of less than 10%. For CLPP, it was employed
240 the correlation dissimilarity (1 - Pearson's correlation index) on the AWCD transformed
241 data (Hackett and Griffiths, 1997).
242 For BCC, CLPP, Nuse and Puse, the consistency of groups of samples according to day
243 and depth of sampling was tested by Non-Parametric Multivariate Analysis of Variance
244 (NPMANOVA) (Anderson, 2001) in software PAST 2.14 (Hammer *et al.*, 2001) with

9,999 permutations and using the same similarity/dissimilarity measures as in the clusters. For Nuse and Puse it was employed the euclidean distance as dissimilarity measure.

The effect of environmental variables (temperature, pH, seston, chlorophyll *a*, LMW substances, NH_4^+, NO_2^-, NO_3^-, PO_4^{-3}) on BCC and CLPP was assessed by canonical correspondence analysis (CCA). For BCC, ribotypes with frequency of occurrence <10% were excluded. The global significance of the CCAs and the significance of the individual axes were tested by permutation ($\alpha = 0.05$, 1,000 permutations). The whole procedure followed Borcard *et al*. (2011) and was performed in the R 3.0.0 environment (R Development Core Team, 2013) using the package Vegan (Oksanen *et al*., 2013). For all significance tests (NPMANOVA and permutation tests) it was adopted a level of $\alpha = 0.05$. All graphical displays were performed in R 3.0.0 (R Development Core Team, 2013).

3. Results

The first day was characterized by mesohaline conditions (salinity 10-17), followed by an intrusion of saltwater in the second day, with polyhaline waters (salinity 21-30) from station B to F (Fig. 2A). The seston varied from a minimum of 14.5 mg L^{-1} in station H at the surface in the first day to a maximum of 72.5 mg L^{-1} at the bottom of station B in the second day (Fig. 2B). The chlorophyll *a* showed a peak of 5.0 µg L^{-1} in the middle of the transect (station F) in the first day, and a second peak of similar magnitude at the bottom of station B in the second day (4.92 µg L^{-1}) (Fig. 2C). The LMW substances displayed higher values at the middle of the transect (peak of 3.9 10^{-2} units of absorbance at 250 nm) in the first day at stations E-F and at the second day the values

increased along the transect towards the inner part of the estuary, peaking at the surface of station I (4.2 10^{-2} units of absorbance at 250 nm) (Fig. 2D). The mean temperature did not change at the surface for both days (mean: 19.3 ºC; range: 18.8-19.5 °C in the first day and 19.0-19.5 °C in the second day) and the mean bottom temperature was slightly lower (mean: 19.2; range: 19.1-19.3 ºC). The pH increased from the first day (mean: 7.9; range: 7.8-8.1) to the second day at the surface (mean: 8.1; range: 7.8-8.3) and at the bottom (mean: 8.2; range: 7.9-8.3).

The concentrations of dissolved inorganic nutrients increased in the second day, with higher values at the bottom. NH_4^+ concentrations were generally low except at the surface of the station A in the first day (1.03 µM) and at station I (0.16 µM) and at the bottom in the middle of the transect (station E, 0.35 µM) in the second day (Fig. 3A). NO_2^- concentrations were also higher in the first day at station A (0.24 µM) at the surface and decreased towards the inner estuary; on the second day this general pattern remained, but with higher values mainly until station D (0.35 µM) at the surface and peak (0.59 µM) at station E at the bottom (Fig. 3B). NO_3^- values were similarly low along the transect at the first day, but increased on the second day at the mouth of the estuary, decreasing towards its interior (surface peak at station B: 0.91 µM and bottom peak at stations D-E: 1.06 µM) (Fig. 3C). PO_4^{-3} concentrations were similar across the transect on the first day, increasing slightly in the inner estuary (peak at station H: 0.41 µM); on the second day, the concentrations increased and presented a peak at station I at the surface (2.12 µM), whereas the higher values at the bottom were found in the middle of the transect (stations C: 0.78 µM and E: 0.74 µM) (Fig. 3D).

The Nuse index was variable on the first day with higher values at both ends of the transect at the surface (station A: 0.28 and station H: 0.24). This index increased both at the surface and the bottom on the second day and tended to decrease towards the

295 interior of the estuary (surface peak at station B: 0.37; bottom peak at station D: 0.41)
296 (Fig. 3E). The Puse index was also variable on the first day at the surface, tending to be
297 higher close to the mouth of the estuary (maximal at station B: 0.06); on the second day,
298 this index displayed higher values and tended to decrease toward the inner estuary
299 (surface peak at station B: 0.07; bottom peak at station E: 0.09) (Fig. 3F).
300

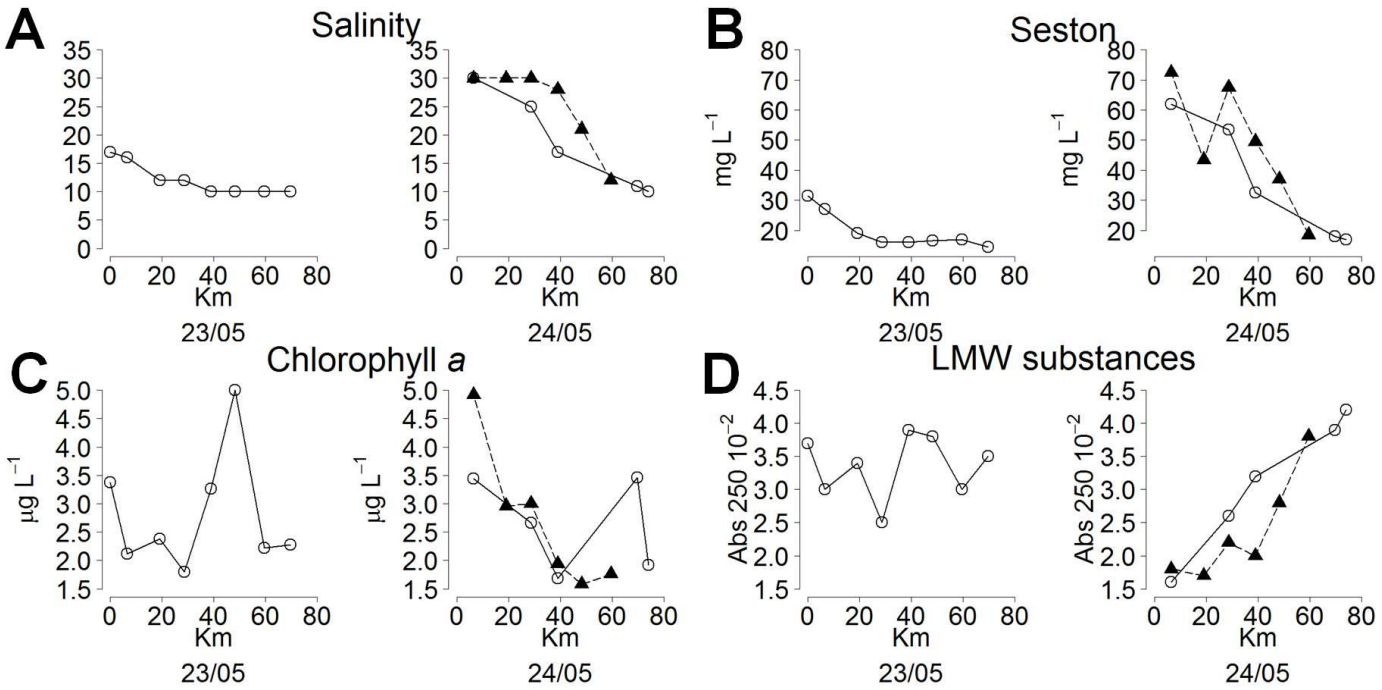


Fig. 2: Main environmental variables along the transect from the estuary mouth (0 km, point A) to the inner limit of the Patos Lagoon estuary (74 km, point I) sampled during May 23 and 24 2012. (—○—), surface samples; (—▲—), bottom samples.

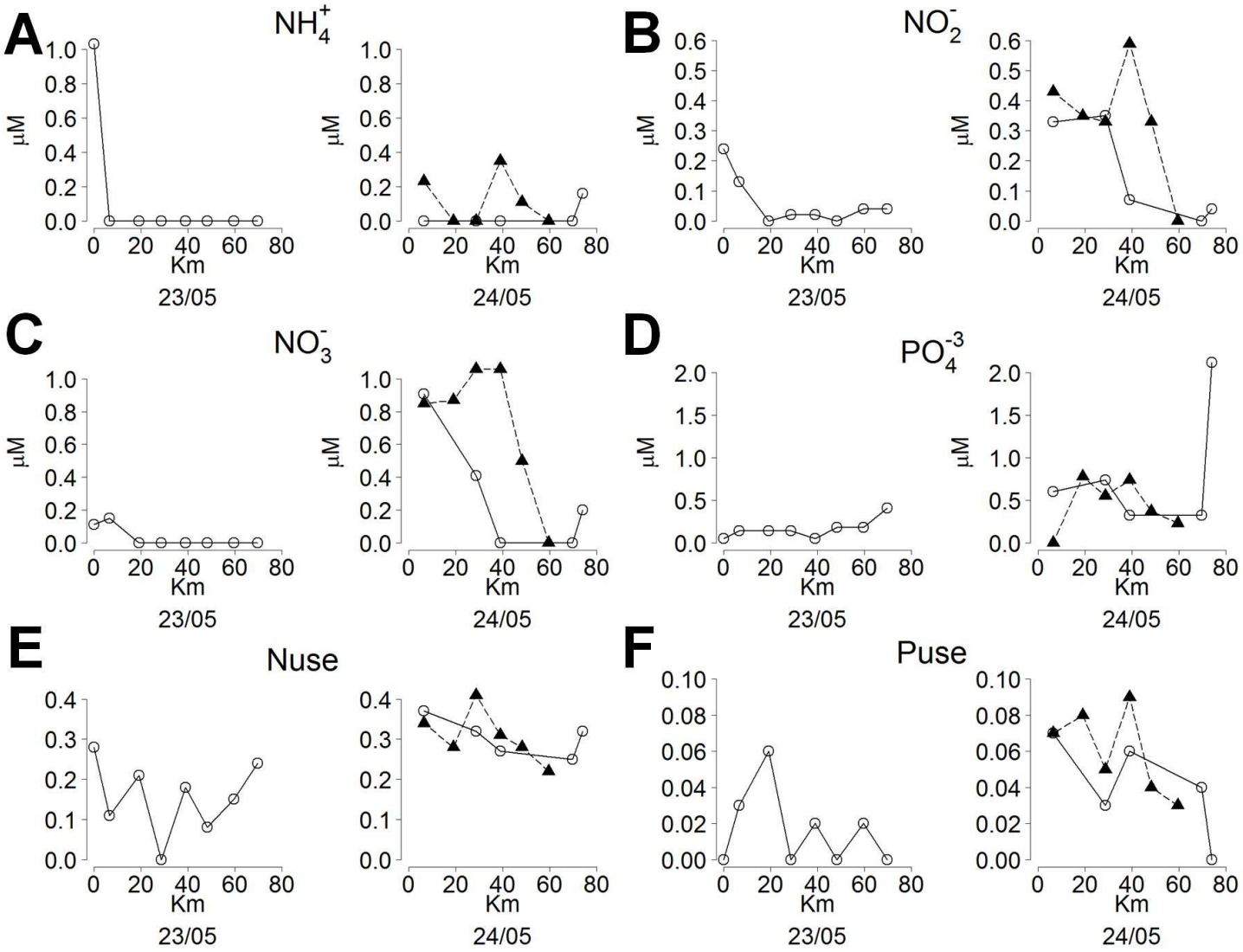


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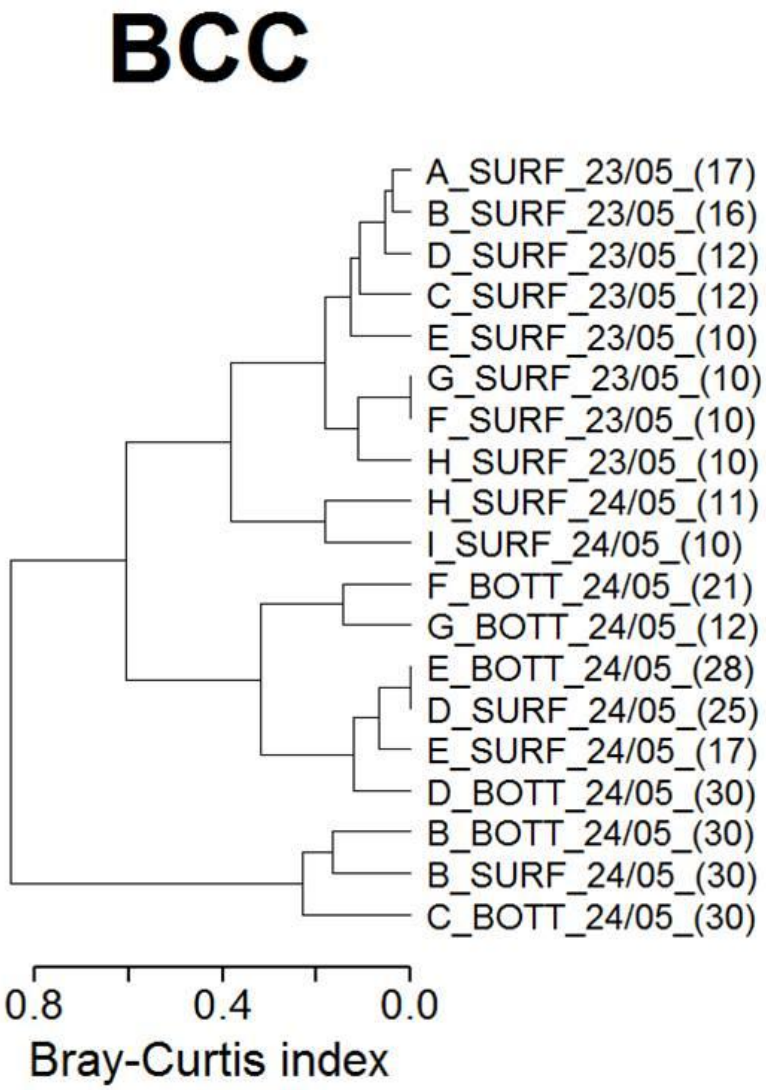
Fig. 3: Dissolved inorganic nutrients and nutrient limitation indicators (Nuse and Puse) along the transect from the estuary mouth (0 km, point A) to the inner limit of the Patos Lagoon estuary (74 km, point I) sampled during May 23 and 24 2012. (–○–), surface samples; (–▲–), bottom samples.

The cluster of BCC revealed a separation of samples mainly by salinity and day of sample. At higher similarities and in many occasions, smaller clusters originated joining close or adjacent stations. At ~85% of dissimilarity two groups split: one containing samples of the second sampling day with salinity 30 (stations B surface and bottom and station C bottom), except station D bottom, which joined the rest of the samples in the other group. This large group split at ~60% dissimilarity in one set of samples of the first day plus the stations H and I (surface), all presenting salinity <17, and other group of samples all of the second day and with salinity between 12 and 30 (Fig. 4).

Most of the ribotypes (60%) were found across all samples, whereas a few (16%) were only found in the upper estuary on the first day and some were found only in the lower to middle estuary (24%) on the second day (Fig. 5).



320

321 **Fig. 4:** Cluster analyses of bacterial community composition (BCC) along the transect

322 (A-I) from the estuary mouth to the inner limit of the Patos Lagoon estuary sampled

323 during May 23 and 24 2012. The salinity of samples is shown inside parentheses.

324

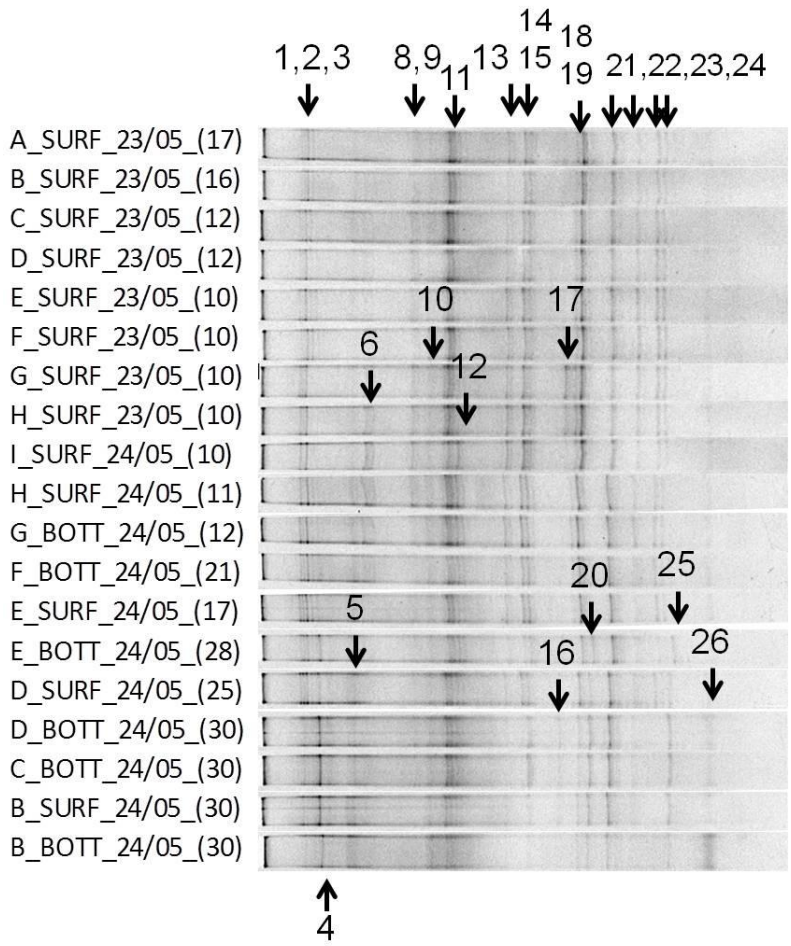


Fig. 5: Band positions detected by TTGE that are common to all samples (rybotypes: 1-3,8-9,11,13,14-15,18-19,21-24), present only in the upper Patos Lagoon estuary (first and second day at stations F-I; rybotypes: 6,10, 12, 17) and present only in the lower-middle estuary at the second day (station B-E; ribotypes: 4,5, 16, 20, 25-26) along the transect from the estuary mouth to the inner limit of the estuary sampled during May 23 and 24 2012. The salinity of samples is shown inside parentheses.

The pattern of CLPP did not display a clear clustering according to day or salinity. The pattern of consumption of organic substrates showed a gradient of lower activity (richness of oxidized substrates) in lower salinity to higher activity in high salinity waters. The first day samples and also station I (surface) of the second day were

337 characterized by consumption of fewer substrates , but some more heavily utilized (> 3
338 times the absorbance divided by AWCD), like carbohydrates (β-methyl D-glucoside, D-
339 mannitol), carboxylic acids (D-galactonic-acid γ-lactone, D-glucosaminic acid, D-malic
340 acid), amino acids (L-arginine, L-serine, glycyl-L-glutamic acid) and polymers (Tween
341 80). The other samples of the second day were characterized by a consumption of a
342 large number of substrates, but less heavily (< 2 times the absorbance divided by
343 AWCD) (Fig. 6).
344 The NPMANOVA revealed significant differences between all samples from the first
345 and the second day for BCC, CLPP, Nuse and Puse, between all surface and bottom
346 samples for BCC, CLPP and Puse and a difference between the surface and bottom
347 samples of the second day for CLPP only (Table 2).
348

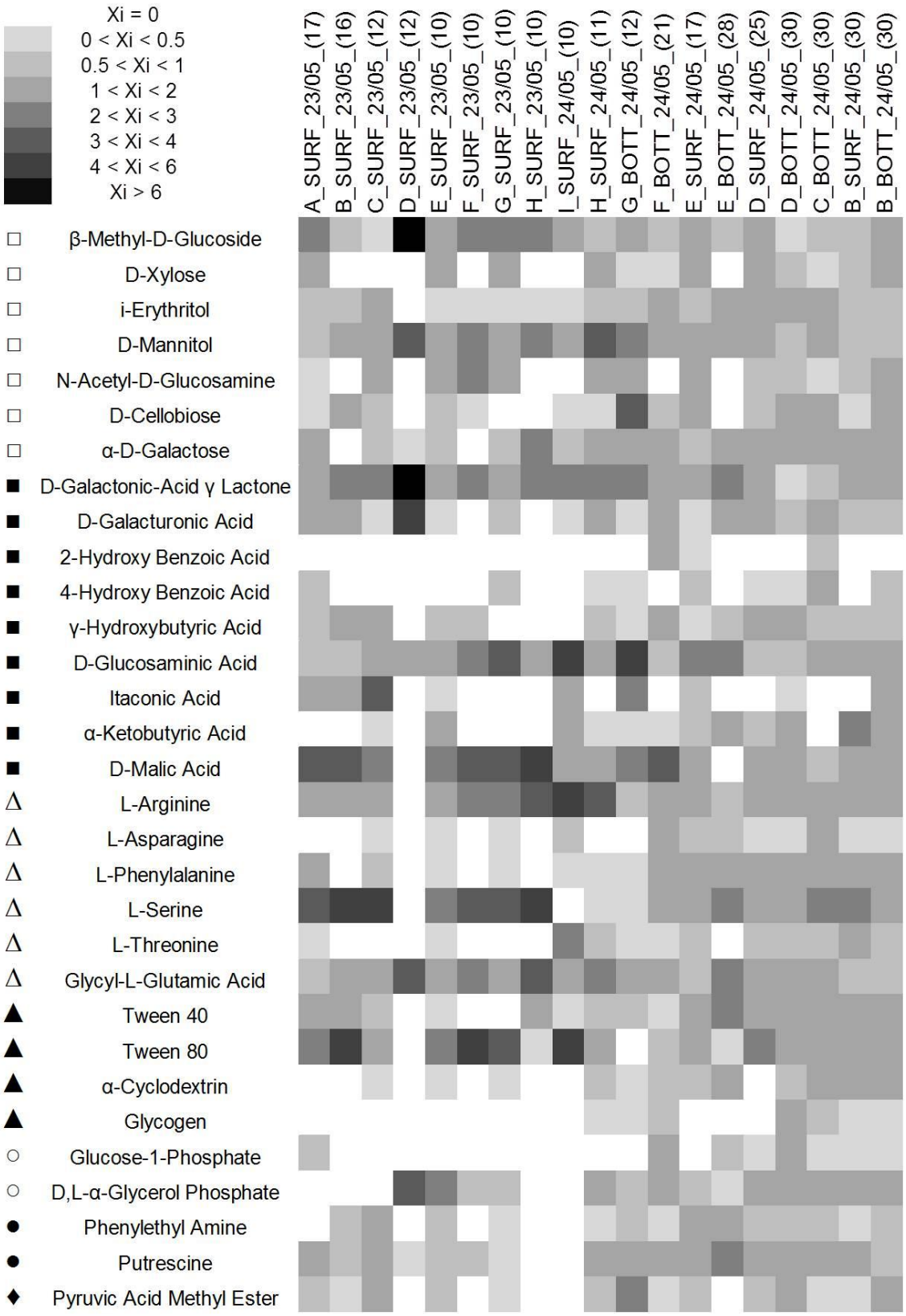

Xi = 0
0 < Xi < 0.5
0.5 < Xi < 1
1 < Xi < 2
2 < Xi < 3
3 < Xi < 4
4 < Xi < 6
Xi > 6
A_SURF_23/05_(17)
B_SURF_23/05_(16)
C_SURF_23/05_(12)
D_SURF_23/05_(12)
E_SURF_23/05_(10)
F_SURF_23/05_(10)
G_SURF_23/05_(10)
H_SURF_23/05_(10)
I_SURF_24/05_(10)
H_SURF_24/05_(11)
G_BOTT_24/05_(12)
F_BOTT_24/05_(21)
E_SURF_24/05_(17)
E_BOTT_24/05_(28)
D_SURF_24/05_(25)
D_BOTT_24/05_(30)
C_BOTT_24/05_(30)
B_SURF_24/05_(30)
B_BOTT_24/05_(30)
β-Methyl-D-Glucoside
D-Xylose
i-Erythritol
D-Mannitol
N-Acetyl-D-Glucosamine
D-Cellobiose
α-D-Galactose
D-Galactonic-Acid γ Lactone
D-Galacturonic Acid
2-Hydroxy Benzoic Acid
4-Hydroxy Benzoic Acid
γ-Hydroxybutyric Acid
D-Glucosaminic Acid
Itaconic Acid
α-Ketobutyric Acid
D-Malic Acid
L-Arginine
L-Asparagine
L-Phenylalanine
L-Serine
L-Threonine
Glycyl-L-Glutamic Acid
Tween 40
Tween 80
α-Cyclodextrin
Glycogen
Glucose-1-Phosphate
D,L-α-Glycerol Phosphate
Phenylethyl Amine
Putrescine
Pyruvic Acid Methyl Ester

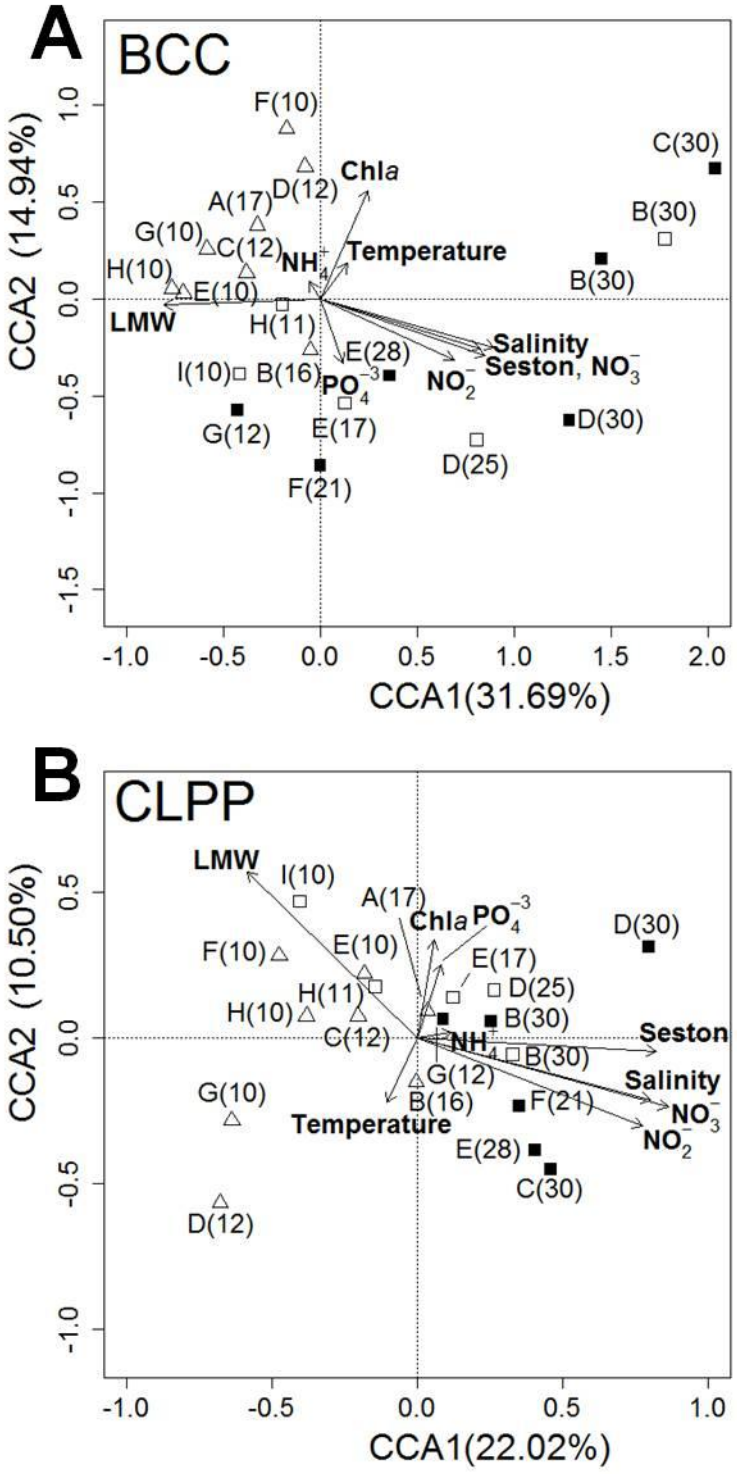
Fig. 6: Pattern of consumption of organic substrates along the transect from the estuary mouth to the inner limit of the Patos Lagoon estuary sampled during May 23 and 24 2012. Xi corresponds to the raw absorbance of each substrate at 595 nm minus the control and divided by the plate's respective AWCD. Classes of substrates are indicated by: (□) carbohydrate, (■) carboxylic acid, (Δ) amino acid, (▲) polimer, (○) phosphorilated chemical, (●) amine, (♦) ester. The salinity of samples is shown inside parentheses.

The environmental variables tested accounted for a significant part of the variation of BCC (*pseudo*-F = 1.68; P-value = 0.024) and CLPP (*pseudo*-F = 1.46; P-value <0.05). For BCC, the total constrained variation was 62.7% and the first and second canonical axes represented 31.2% (*pseudo*-F = 7.65; P-value < 0.01) and 14.9% (*pseudo*-F = 3.6; P-value < 0.05) of the total constrained variation, respectively. In general the distribution of stations was associated to higher salinity, seston, NO_3^- and NO_2^- on one side and lower LMW substances on the other of the first CCA axis. The distribution of stations considering the Axis 2 showed an inverse relationship between chlorophyll *a* and temperature and PO_4^{-3} (Fig. 7A).

For CLPP the total constrained variation was 59.3% and the first and second canonical axes represented 22% (*pseudo*-F = 4.87; P-value < 0.01) and 10.5% (*pseudo*-F = 2.32; P-value < 0.05) of the total constrained variation, respectively. In general the samples distribution was associated to the same variables as for BCC, i.e., higher salinity, seston, NO_3^- and NO_2^- and were related to lower LMW substances on the first CCA Axis, whereas higher chlorophyll *a* and PO_4^{-3} were inversely related with temperature along the second CCA axis (Fig. 7B).

Table 2: Differences between day and depth of sampling assessed by Non-parametric MANOVA (NPMANOVA) based on bacterial community composition (BCC), community level physiological profiles (CLPP), Nuse and Puse indexes.

Groups	**BCC**		**CLPP**		**Nuse**		**Puse**	
	***Pseudo*-F**	**P-value**	***Pseudo*-F**	**P-value**	***Pseudo*-F**	**P-value**	***Pseudo*-F**	**P-value**
May 23 × May 24 (all samples, n = 19)	**11.35**	**<0.001**	**2.89**	**<0.05**	**21.14**	**<0.001**	**0.31**	**<0.05**
Surface × Bottom (all samples, n = 19)	**4.39**	**<0.05**	**3.86**	**<0.01**	-0.03	0.53	**7.57**	**<0.05**
Surface × Bottom (day 24, n = 6)	0.66	0.49	**2.64**	**<0.05**	0.003	0.96	1.77	0.22



383

384 **Fig. 7:** Canonical correspondence analysis (CCA) of bacterial community composition
385 (BCC) and community level physiological profiles (CLPP) for samples collected along
386 the transect from the estuary mouth to the inner limit of the Patos Lagoon estuary during
387 May 23 and 24 2012. Samples are indicated by capital letters with respective salinities

388 inside parentheses. (Δ) surface samples, first day; (□) surface samples, second day; (■)
389 bottom samples, second day.
390
391 **4. Discussion**
392
393 *4.1 Environmental Conditions*
394
395 Our study showed a short-term (~1 day) variation in environmental conditions
396 associated to the entrance of saltwater in the estuary. Saltwater intrusion occurred on the
397 second day, with the saltwedge causing sediments re-suspension and increase of
398 nutrients, as previously observed in the Patos Lagoon estuary mouth (Kantin and
399 Baumgarten, 1982; Hartmann and Schettini, 1991). However, the augment of nutrients
400 (NO_2^-, NO_3^- and PO_4^{-3}) concentration after the entrance of coastal water has been
401 attributed to the displacement of nutrient-rich interstitial water by high density saltwater
402 (Abreu et al., 1995a) and to the return of previously exported estuarine water
403 (Niencheski *et al*., 1988; Abreu et al., 1995a). Both processes may occur simultaneously
404 and affect different portions of the estuary.
405 Similarly, higher chlorophyll *a* values measured at the bottom of the station B during
406 the second day was probably generated by the re-suspension of microalgae by the
407 saltwedge entrance, as previously observed by Abreu *et al*. (2010). LMW substances,
408 however, were lower in saltwater and increased towards the inner estuary.
409
410 *4.2 Bacterial Community Composition (BCC)*

411 The CCAs revealed that the shifts in environmental conditions led to shifts in BCC and
412 CLPP between the two sampling days and between surface and bottom samples, which
413 was confirmed by NPMANOVA.
414 The cluster analysis revealed a clear separation of samples by days, mainly reflecting
415 the increase in salinity in the second day, but also the increase of inorganic nutrients and
416 seston in the same period. The effect of salinity is expected, given the well recognized
417 difference in bacterial community compositions of fresh- and saltwater (Glöckner *et al*.,
418 1999). The sub-group formed by the three samples located close to the estuary mouth
419 (stations B surface and bottom, C bottom) with salinity 30, reinforces the idea of a
420 saltwater bacterial community. Seston re-suspension and higher chlorophyll a and
421 nutrient load measured in this region may also have influenced the results.
422 The increase in seston may have contributed to higher number of attached bacteria,
423 which respond to different environmental conditions than free-living bacteria (Jing and
424 Liu, 2012). The BCC is also known to change over gradients of primary productivity
425 (Horner-Devine *et al*., 2003); and it has been demonstrated experimentally that bacteria
426 develop completely different phylogenetic composition when growing on either low or
427 high molecular weight dissolved organic carbon, in spite of deriving from the same
428 inoculum (Covert and Moran, 2001). Higher bacterial abundance and production were
429 also measured at stations in the estuarine plume where phytoplankton presented higher
430 biomass and primary production rates in the Patos Lagoon estuary (Abreu et al. 1995b).
431 Bacteria actively uptake dissolved inorganic nutrients (Kirchman, 1994) and it has been
432 demonstrated experimentally that inorganic nutrient enrichment can lead to the
433 development of different bacterial communities in comparison to nutrient-poor
434 treatments (Schäfer *et al*., 2001; Leflaive *et al*., 2008). Growth of bacteria in such a
435 short time is feasible, since bacterial community doubling rates of < 1 day have been

436 estimated in estuaries (Crump *et al*., 2004), with significant shifts of BCC within tidal
437 cycles (Chauhan *et al*., 2009). The contribution of bacteria that entered the estuary with
438 coastal water also contributed to the community, since an important fraction of the
439 ribotypes detected (24%) were found only in the middle to lower estuary on the second
440 day.
441 The depth significantly influenced BCC due to stratification, since stations that
442 displayed stratification (D and E, second day) also showed distinct bacterial
443 composition in the bottom and surface, whereas at station B (second day) where the
444 water-column was mixed, bottom and surface samples grouped closely together. The
445 vertical variation of bacterial diversity in the water column has been reported for other
446 systems like lakes (Humayoun *et* al., 2003; Wever *et al*., 2005; Shade *et al*., 2008) and
447 the ocean (Ghiglione *et al*., 2008; Fortunato and Crump, 2011; Fortunato *et al*., 2012),
448 where differences have been attributed to the physical barrier of the thermocline. In the
449 Patos Lagoon estuary, however the halocline may be a temporary and weak barrier.
450
451 *4.3 Community level physiological profiles (CLPP)*
452 The pattern observed for CLPP in this study contrasts with those found previously in the
453 Patos Lagoon estuary (They *et al*., unpublished results), when a lower richness but
454 heavier consumption of specific compounds in the classes of carbohydrates, carboxylic
455 acids, amino acids and polymers was found in high salinity waters (They *et al*.,
456 unpublished results). Accordingly, Schultz and Ducklow (2000) and Thottahthil et al.
457 (2008) also found a decrease in activity (lower overall consumption of Ecoplate
458 substrates) towards higher salinity waters in different estuaries.
459 In our previous studies the low salinity waters were associated to high concentrations of
460 dissolved inorganic nutrients (They *et al*., unpublished data), different from the

461 observed in this work. This suggests that the CLPP respond primarily to the availability
462 of dissolved inorganic nutrients and secondarily to salinity.
463 It is known that inorganic nutrients do affect bacterial metabolism; it has been
464 demonstrated experimentally in terms of thymidine incorporation by different groups of
465 bacteria (Boucher *et al*., 2006) and CLPP (Leflaive *et al*., 2008). Another important
466 aspect of bacterial activity is that CLPP displays high plasticity, resulting in different
467 responses to phosphorus and nitrogen availability even for similar bacterial community
468 composition (Leflaive *et al*. 2008). As a result, CLPP (Thottathil *et al*., 2008), bacterial
469 production and organic matter decomposition (Lemke *et al.*, 2010) can vary
470 significantly within short-term scales (Thottathil *et al*., 2008; Chauhan *et* al., 2009;
471 Lemke *et al.*, 2010).
472 The increase in Nuse and Puse indexes on the second day was unexpected, since it
473 contrasts with the increase in nutrients in this period. The Nuse index indicates states of
474 nitrogen limitation (Sala *et al*., 2006), whereas Puse has been also linked to phosphorus
475 limitation in the Patos Lagoon estuary (They *et al*., unpublished results). In addition to
476 variation in CLPP response due to the metabolic plasticity of bacteria (Leflaive *et al*.,
477 2008), it is possible that the response of bacteria to the increase in nutrients display a
478 lagged response higher than the time span of the present study, since it has been
479 reported that nutrient limited bacterial communities display lag response in Ecoplate in
480 comparison to bacteria from nutrient rich environments (Leflaive *et al*., 2008).
481 Additionally, Lyons and Dobbs (2012) have found higher Nuse index for aggregate
482 bacteria in comparison to free-living bacteria, despite deriving from the same inoculum
483 and experiencing the same nutrients levels. They concluded these two communities
484 impacted biogeochemistry differently and that the extent of this impact should be
485 affected to their relative proportion in the system.

The effect of seston may also be significant, as it has been demonstrated that bacteria in aggregates display higher richness of oxidized Ecoplate substrates in comparison to free-living bacteria, which in turn oxidized smaller number of substrates, with heavier consumption (Lyons and Dobbs, 2012). This response parallels the pattern found in the Patos Lagoon estuary and could partly explain differences found between the first and second days of sampling, probably with different contributions of free and attached bacteria.

5. Conclusions

Overall our results support the general view that a large proportion of bacterial community composition is structured mainly by salinity and secondarily by other environmental variables like nutrients (van der Gucht *et al*., 2007). Unexpectedly, the bacterial activity was higher in high salinity waters, associated to higher nutrients content in these waters. This pattern led us to conclude that activity may respond primarily to the availability of dissolved inorganic nutrients and only secondarily to salinity. This finding has important implications since it deviates from the common idea of lower bacterial activity in high salinity. However other field and experimental studies must be carried on in order to depict a better picture of bacterial composition and activity in this estuary

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512

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