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DINÂMICA FUNCIONAL DA COMUNIDADE MICROBIANA HETEROTRÓFICA EM LAGOA RASA SUBTROPICAL

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Porto Alegre, junho de 2011

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Dissertação apresentada ao Programa de Pós-Graduação em Ecologia, do Instituto de Biociências da Universidade Federal do Rio Grande do Sul, como parte dos requisitos para obtenção do título de Mestre em Ecologia.

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“Descobrir consiste em ver o que todos viram e pensar o que ninguém pensou.”

Albert Szent Gyorgy

“Nada da vida é para temer. É para ser entendido.”

Marie Curie

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RESUMO

Ecossistemas aquáticos flutuam em torno de tendências, podendo ocorrer transições súbitas de um regime persistente para outro, alterando a viabilidade dos recursos ou dos parâmetros físico-químicos. Seguindo essa tendência, comunidades variam no tempo e no espaço como resultado de suas interações com o ambiente e com os outros organismos. Comunidades microbianas aquáticas são importantes componentes do metabolismo aquático, atuando na reciclagem de nutrientes pela remineralização e na transferência de biomassa pela alça microbiana. A compreensão da dinâmica funcional microbiana em lagos é uma importante ferramenta para o entendimento desses sistemas, uma vez que a funcionalidade e a composição microbiana podem refletir as condições gerais da lagoa em questão. Desse modo, no presente trabalho, foi utilizada a abordagem de impressões metabólicas da comunidade microbiana na Lagoa Mangueira, uma grande lagoa costeira, rasa e subtropical localizada no extremo sul do Brasil. Teve como objetivo avaliar, pioneiramente para essa região, a diversidade metabólica microbiana aquática através de padrões de consumo de fontes de carbono disponíveis, utilizando Biolog Ecoplates^{TM} e verificar a existência de dinâmicas temporais e espaciais na preferência de consumo desses substratos. Foi observada heterogeneidade temporal e espacial na preferência de consumo de substratos ao longo da Lagoa. Tais preferências puderam ser representadas por diferentes substratos indicadores, associados às estações e aos locais da Lagoa. O consumo das fontes de carbono esteve relacionado com a variabilidade ambiental de fatores como turbidez, transparência da água, nutrientes, clorofila *a*, carbono orgânico temperatura. Isso evidencia que a dinâmica funcional foi influenciada pela dinâmica de nutrientes, pelos componentes de produtividade, pela sazonalidade e pela compartimentação da Lagoa. Nesse sentido, a investigação do consumo de fontes de carbono, no presente estudo, se mostrou um bom indicador da dinâmica funcional microbiana para ecossistemas aquáticos.

Palavras-chave: *substratos de carbono, assembléias microbianas heterotróficas aquáticas, diversidade funcional, lagoa costeira rasa subtropical, padrão temporal e espacial.*

ABSTRACT

Aquatic ecosystems float around trends in which abrupt transitions can occur between persistents regimes, alterning the viability of the resources or the physical and chemical parameters. Following this trend, communities vary in time and space as a result of their interactions with the environment and other organisms. Microbial communities are important components in the aquatic metabolism, responsible for recycling of nutrients by remineralization, and transfering of biomass through the microbial food web. Understanding the functional microbial dynamics in lakes is an important tool to understand these systems, since the microbial composition and function may reflect the overall condition of the lake. The approach of metabolic fingerprint of microbial communities in shallow lakes was applied in the current study in Lake Mangueira, a costal and large subtropical shallow lake located in southern Brazil. The main goal was to evaluate, pioneered for this region, the heterotrophic microbial metabolic diversity through consumption patterns of available carbon sources using Biolog Ecoplates^{TM} and verify the existence of temporal and spatial dynamics of consumption preference in these substrates. As a result, was observed temporal and spatial heterogeneity of substrate consumption preference among the Lake. Such preferences were represented by different substrate indicators, associated with seasons and sites within the Lake. The substrate utilization was related to environmental variability of factors as turbidity, water transparence, nutrients, chlorophyll *a*, organic carbon, water temperature. This is evidence that the functional dynamic was influenced by nutrients dynamic, production component, seasonality and compartimentation. In this way, carbon source utilization approach was a good indicator of functional dynamics in the present study to aquatic ecosystems.

Keywords: *carbon source, aquatic heterotrophic microbial assembly, functional diversity, subtropical shallow lake, temporal and spatial pattern.*

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LISTA DE ABREVIATURAS E SIGLAS

AWCD - *Average Well Color Development* (Média de desenvolvimento de cor)

Chla - Chlorophyll *a* (Clorofila *a*)

CI – *Curve Integration* (Integração da Curva)

CLPP – *Community Level Physiological Profile* (Perfil Fisiológico em Nível de Comunidade)

DIC – *Dissolved Inorganic Carbon* (Carbono Inorgânico Dissolvido)

DOC – *Dissolved Organic Carbon* (Carbono Orgânico Dissolvido)

IC – *Inorganic Carbon* (Carbono Inorgânico)

rRNA – *Ribosomal Ribonucleic Acid* (Ácido Ribonucléico Ribossomal)

TC – *Total Carbon* (Carbono Total)

μL – Microlitros (10^{-6} L)

μm – Micrômetros (10^{-6} m)

MO – Matéria Orgânica

MOD - Matéria Orgânica Dissolvida

NH_4^- - Nitrogênio amoniacal

NO_3^- - Nitrato

nm – Nanômetros (10^{-9} m)

OD – *Optical density* (Densidade Óptica)

PB – Produção Bacteriana

PO_4^{-3} - *Soluble Reactive Phosphorus* (Fósforo Solúvel Reativo)

PP- Produção Primária

PSUE - *Proportional Substrate Utilization Efficiency* (Eficiência Proporcional de Utilização de Substrato)

RB – Respiração Bacteriana

TN – *Total Nitrogen* (Nitrogênio Total)

TOC- *Total Organic Carbon* (Carbono Orgânico Total)

TP - *Total Phosphorus* (Fósforo Total)

TSS - *Total Solid Suspensed* (Sólidos Suspensos Totais)

Turb - Turbidity

OTU – *Operational Taxonomic Unit* (Unidade Taxonômica Operacional)

SUMÁRIO

1 Introdução Geral

1.1 ECOLOGIA MICROBIANA AQUÁTICA

Por muito tempo, a ecologia microbiana ficou limitada ao estudo dos micro-organismos em meios de cultivo. Há cerca de duas décadas, conceitos de estudos moleculares em nível de comunidades microbianas naturais revolucionaram os estudos em ecologia microbiana, permitindo de forma mais acurada, identificar organismos que eram antes considerados pequenos pontos ao microscópio (Fuhrman e Steele, 2008). A partir de então, essa ecologia vem investigando quem são, como estão distribuídos no tempo e no espaço e que funções esses micro-organismos desempenham no ambiente.

Ao estudo de micro-organismos aquáticos, incluem-se bactérias, archea, protistas e fungos, os quais dominam os hábitats aquáticos, tanto numericamente quanto bioquimicamente (Hahn, 2006). Tais micro-organismos respondem rapidamente a mudanças ambientais e são os grandes responsáveis por processos biogeoquímicos (metabolização do carbono orgânico dissolvido e ciclo do nitrogênio, por exemplo) que são cruciais para o funcionamento do ecossistema (Allan, 1995; Hahn, 2006).

A estrutura e função desses micro-organismos aquáticos são influenciadas, tanto por fatores extrínsecos (regionais – impõem sincronia na dinâmica de vários parâmetros do ecossistema), quanto por fatores intrínsecos (local-específicos – interações tróficas, dinâmica da população e características internas) (Kent *et al.*, 2007).

Desta forma, é possível elencar alguns tópicos que se fazem relevantes aos estudos em ecologia microbiana.

1.1.1 Comunidade microbiana - definição

Por comunidades microbianas se entende a assembléia de diferentes espécies de micro-organismos, filogeneticamente relacionados (FAUTH *et al*., 1995), em diferentes proporções, ocorrendo em sincronia no tempo e no espaço, e realizando diversas funções. Refletem as propriedades dos indivíduos mais as propriedades de suas interações (Begon *et al.*, 2006). Tais comunidades incluem uma gama de micro-organismos procariotos e eucariotos. Dependendo do ambiente aquático, as bactérias podem representar mais de 90% desses micro-organismos (Hahn, 2006) .

Estima-se que a diversidade microbiana exceda a diversidade de plantas e animais. A ausência de conhecimento detalhado sobre essa diversidade resulta na perda de informações importantes sobre a vida no planeta, dada a abundância desses micro-organismos e a importância que têm nas transformações biogeoquímicas (Whitman *et al.*, 1998). A diversidade microbiana inclui a composição genética dos microrganismos, e seu papel ecológico ou funcional dentro do ecossistema (Hunter-Cevera, 1998). A árvore filogenética universal dos seres vivos, baseada na comparação de sequências do 16S e do 18S rRNA (Madigan *et al.,* 2004), apresenta três grandes domínios de seres vivos: (i) domínio bactéria e (ii) domínio archea correspondendo aos micro-organismos procariontes, como bactérias e archeas; e (iii) domínio eucarya, representado por micro-organismos eucariontes, como algas microscópicas, fungos filamentosos, leveduras, protozoários e ciliados (Madigan *et al.,* 2004).

1.1.2 Interações tróficas e Importância

Por um longo período em estudos ecológicos foi atribuído, à comunidade microbiana, o papel de meros decompositores da matéria orgânica. No entanto, vem

sendo discutido, o reconhecimento dos micro-organismos como os principais consumidores de energia dos ecossistemas (Pomeroy, 1974; Fuhrman e Azam, 1982).

Na evolução deste pensamento, Azam *et al*., (1983) propuseram que micro-organismos pelágicos formam uma Alça Microbiana (*Microbial Loop*) dentro da rede trófica pelágica, na qual as bactérias, que utilizam a matéria orgânica dissolvida na coluna d'água como fonte de energia, são predadas e consumidas por protozoários que são predados pelo microzooplâncton. É através desse elo que grande parte da regeneração de nutrientes não é mais atribuída diretamente às bactérias, mas sim aos protozoários que as consomem e liberam o excesso de amônia e fosfato, tanto em ecossistemas aquáticos como terrestres (Pomeroy *et al*., 1988). Desta forma, a matéria orgânica também retorna à teia trófica clássica, e então ocorre a ciclagem dos nutrientes em ecossistemas aquáticos (Azam *et al*., 1983; Sherr e Sherr, 1988).

Na relação entre a produção (PB) e respiração (RB) bacteriana e a produção primária fitoplanctônica (PP) de carbono orgânico dissolvido, o metabolismo bacteriano é dependente de fontes liberadas por fitoplâncton. Segundo Fuhrman e Azam (1982), comunidades microbianas heterotróficas podem consumir mais de 60% do total da produção primária na coluna d'água. Conhecimentos acerca desses fatores são pré-requisitos para a compreensão da transformação e mineralização da matéria orgânica, assim como do estado trófico do sistema (Thottathil et al., 2008a).

A matéria orgânica absorvida pelas bactérias também pode ser respirada, situação em que ocorre a mineralização do carbono orgânico dissolvido para CO_2 (Cotner e Biddanda, 2002). Estima-se uma linearidade da respiração bacteriana com sua taxa de crescimento, na qual a proporção do carbono absorvido que é respirado seja de 40 a 90% (Azam *et al*., 1983). Então, em situações de PP/RB<1, a respiração suplanta a produção primária (Del Giorgio e Peters, 1993; Del Giorgio *et al*., 1997). Esta

heterotrofia líquida, em sistemas improdutivos, é sugerida como a causa de supersaturação de CO_2 (Kling *et al*., 1992; Cole *et al*., 1994), tornando lagos/lagoas fontes líquidas de CO_2 para a atmosfera.

Neste contexto é que micro-organismos pelágicos são organismos chave no metabolismo dos ecossistemas aquáticos atuando tanto no fluxo de energia quanto na ciclagem da matéria. E essa importância parece ser mais significativa em sistemas oligotróficos (pouco produtivos) e diminuída em sistemas eutróficos (mais produtivos) (Baines e Pace, 1991; Burns e Galbraith, 2007). Ainda, micro-organismos heterotróficos são elementos-chave em áreas de lagos rasos caracterizados pela grande acumulação de matéria orgânica no sedimento (Danovaro e Pusceddu, 2007), uma vez que eventos climáticos e a hidrodinâmica - característico desses locais - podem disponibilizar uma grande fração de carbono orgânico para a coluna d'água e, consequentemente, para os micro-organismos.

O papel ecológico desses micro-organismos, reduzindo o excesso de carga orgânica, e produzindo biomassa que pode ser usada pelos consumidores, representa um exemplo de mecanismo de *feedback*, prevenindo colapsos no funcionamento desses sistemas lagunares (Danovaro e Pusceddu, 2007).

1.1.3 Forçantes das Comunidades Microbianas Aquáticas

Uma questão central em ecologia é entender os fatores envolvendo mudanças ambientais e os custos que essas mudanças promovem na assembléia de organismos. Mudanças na concentração de importantes fatores ambientais podem alterar a estrutura da teia trófica e a abundância dos organismos-chave do ecossistema (Tammert *et al*., 2005).

A dinâmica do bacterioplâncton em lagos é controlada por importantes fatores como biogeografia (Haig-They *et al.*, 2010), suprimento de substrato, temperatura da água, química da água, composição de fitoplâncton (Burns e Galbraith, 2007), perdas decorrentes da predação (Corno e Jürges, 2008) parasitismo e sedimentação (Weisse, 2005).

A produção bacteriana em diferentes substratos ainda pouco compreendida. No entanto, o grau de diagênese (Farjalla *et al*., 2006) e a utilização preferencial de diferentes componentes da matéria orgânica dissolvida (MOD) por certos grupos de bactérias, revelam que o suprimento de carbono afeta a estrutura da comunidade bacteriana, uma vez que o metabolismo da comunidade é sempre acompanhado por mudanças na sua composição (Findlay *et al*., 2003). Neste contexto, Riemann e Winding, (2001) ainda ressaltam que forças seletivas presentes no microambiente de partículas orgânicas podem governar a sucessão da comunidade em relação à composição filogenética, uma vez que durante afloramentos de fitoplâncton, bactérias agregadas às partículas orgânicas podem ser filogeneticamente distintas das células bacterianas livres na água (Bidle e Fletcher, 1995; Rath *et al*., 1998).

Variações na temperatura e nos nutrientes inorgânicos e orgânicos podem limitar a atividade e a abundância bacteriana, conforme já observado por Vrede (2005). A limitação da produção bacteriana por diferentes elementos pode acarretar em alterações tanto na forma quanto no tamanho bacteriano e, então, desfigurar padrões de forrageio sobre a bactéria e, por consequência, modificar a estrutura da comunidade bacteriana (Vrede *et al*., 2002; Vrede, 2005).

Outra situação, observada por Corno e Jürgens (2008), são as perdas de números bacterianos decorrentes da predação, também influenciando alterações estruturais e funcionais na comunidade ao longo do gradiente de produtividade. Neste estudo, Corno

e Jürgens (2008) observaram que a comunidade bacteriana, apesar de ter sofrido perdas significativas por predação, parece ter aumentado sua produtividade ao longo do gradiente. Devido provavelmente ao aparecimento de morfotipos bacterianos não comestíveis, que antes da predação sofriam a pressão da exclusão por competição com outros morfotipos bacterianos.

Nesse sentido, a disponibilidade de substratos e o forrageio de predadores são duas forças importantes, cuja interação promove regulação da estrutura e função dessas comunidades (Corno e Jürgens, 2008). Essas forças remetem aos controles de regulação ascendente (*Bottom-up*) e descendentes (*Top-down*), respectivamente. Pace e Cole (1994) e Thingstag e Lignell (1997) indicam que o metabolismo bacteriano é principalmente afetado pelo suprimento de substratos, enquanto que os números bacterianos e a biomassa são controlados pelos predadores.

Neste cenário, Gasol *et al*. (2002) acrescentam que comunidades bacterianas são fortemente controladas pela pressão de predação (*top-down*) em ambientes oligotróficos, todavia, à medida que a produtividade aumenta, o controle *bottom-up* assume o papel na regulação da comunidade. Tal relação remete à evidência de *trade-off*, entre a resistência à predação e a habilidade competitiva, explicando tais padrões de interação (Hahn e Höfle, 2001). Assim, uma vez que grupos bacterianos são mantidos em baixos níveis de abundância, dada a situação de competição, passam a aumentar sua abundância e, então alterar padrões de consumo de MOD, quando ocorre a predação sobre o outro grupo bacteriano.

1.2 LAGOA MANGUEIRA, RASA E SUBTROPICAL – SISTEMA HIDROLÓGICO DO TAIM – DINÂMICA MICROBIANA

Ambientes costeiros rasos, como lagoas costeiras, normalmente são sistemas altamente produtivos e dominados por macrófitas aquáticas onde o acúmulo de matéria orgânica no sedimento excede a capacidade de seu consumo (Danovaro e Pusceddu, 2007).

Lagoas costeiras e rasas de grande extensão, como a Lagoa Mangueira, não apresentam significativa estratificação ao longo da coluna d'água e, frequentemente, são caracterizadas por fortes diferenças, de correntes e de plâncton ao longo do gradiente horizontal entre zonas pelágicas e limnéticas (Fragoso Jr. *et al.*, 2008). Esse tipo de lagoa costuma ser fortemente dominada pelo vento, o qual cria condições de heterogeneidade temporal e espacial das características da água, afetando concentrações de nutrientes, promovendo ressuspensão da matéria orgânica do sedimento (Scheffer, 1998) e influenciando as dinâmicas ecológicas entre os diferentes níveis tróficos, (e.g. Carrick *et al*., 1993; Cardoso e Motta Marques, 2009; Rodrigues, 2009).

A Lagoa Mangueira está interconectada ao Banhado do Taim, formando o Sistema Hidrológico do Taim - SHT. Esse sistema é drenado pela Lagoa Mirim e caracterizado por uma série de áreas alagáveis caracterizadas por áreas úmidas (banhados) subtropicais associadas a lagoas de água doce, em uma dinâmica de baixo relevo marginal ao Oceano Atlântico (Fig. 1).

A região do SHT, onde se insere a Lagoa Mangueira, é caracterizada por um clima subtropical úmido, apresentando as quatro estações bem definidas (Cfa; Koöppen, 1936). Sistemas aquáticos localizados em áreas mais aquecidas, tipicamente se diferenciam de lagos temperados em muitos aspectos relacionados às interações e dinâmicas das comunidades (Mefford *et al*., 2007).

Os banhados e lagos rasos associados são ecossistemas de grande importância ecológica. Constituem um complexo mosaico de habitats, com diferentes estruturas, funções e valores característicos, podendo variar no tempo e no espaço. Considerando que diversos parâmetros podem atuar em sincronia com comunidades microbianas, influenciando sua estrutura e sua atividade, a dinâmica da comunidade bacteriana de lagos pode variar temporalmente e espacialmente dentro de habitats (Lindström, 1998), bem como entre habitats (Yannarell e Triplett, 2004).

Essa premissa cria a perspectiva de heterogeneidade funcional dos organismos planctônicos (Kolasa e Rollo, 1991), que é o resultado da interação ecológica entre os organismos e seu ambiente. Assim, os organismos percebem a variação do habitat e realizam suas funções em resposta aos fatores ambientais. Nesse sentido, fatores abióticos tornam-se importantes determinantes da estrutura microbiana no espaço (Pinel-Alloul e Ghadouani, 2007) e ao longo do tempo.

1.3 ABORDAGEM DE PERFIL FUNCIONAL MICROBIANO – IDÉIAS CENTRAIS

Por muito tempo, estudos sobre micro-organismos estiveram limitados na sua identificação através do desenvolvimento de culturas em laboratório. Este fato representou uma limitação para o desenvolvimento das pesquisas em ecologia microbiana, uma vez que esta técnica permite a identificação de apenas uma fração da vida microbiana (Green e Bohannan, 2006; Hahn, 2006), não sendo suficiente para acessar a diversidade e a funcionalidade em amostras naturais.

O advento de novas técnicas moleculares, no entanto, abriu uma nova perspectiva nas pesquisas de diversidade e função de comunidades microbianas em águas interiores (Hahn, 2006). Em relação à análise de Unidades Taxonômicas

Operacionais (OTUs – unidade de diversidade) para acessar a diversidade genética, muitos progressos têm ocorrido com as metodologias atuais de *molecular fingerprints*. Nesse sentido, Olsen *et al*. (1986) e Pace *et al*. (1986) introduziram o conceito do estudo da composição natural das comunidades microbianas através do seqüenciamento de genes obtidos diretamente da biomassa ao invés de culturas. Entretanto, essas técnicas ainda trazem poucas informações acerca das características fisiológicas em nível de comunidade, ou seja, a "impressão funcional" (*metabolic fingerprints*) dessas comunidades (Miguel *et al.*, 2007). Dessa forma, um dos grandes desafios em ecologia microbiana ainda é a determinação não apenas de qual organismo está presente no ambiente, mas que papel está desenvolvendo nesse ecossistema (Fuhrman e Steele, 2008).

Considerando as peculiaridades elencadas, anteriormente, para caracterizar o papel ecológico e a relação com frações dissolvidas do carbono das comunidades microbianas, principalmente de bactérias, pode-se concluir que tais micro-organismos constituem um importante componente do sistema aquático (Pace e Cole, 1996). No sentido em que conhecimentos sobre a funcionalidade bacteriana são significativos para o entendimento do funcionamento de sistemas aquáticos, bem como para o estudo de teias alimentares aquáticas (Pace e Cole, 1994).

A maioria das bactérias possui metabolismo heterotrófico, ou seja, micro-organismos que dependem exclusivamente ou preferencialmente de compostos orgânicos reduzidos como fonte de energia (organotrofia) para seu desenvolvimento (Pace, 1997; Thomaz, 1999). A partir dessa consideração teórica, a diversidade funcional da comunidade microbiana, acessada pelo consumo de substratos de carbono disponíveis, tornou-se o objeto de interesse do presente trabalho.

Preston-Mafhan *et al*. (2002) conceituam diversidade funcional como o potencial de atividade da comunidade, ou seja, a capacidade dessa comunidade de adaptar seu metabolismo, sua composição e tamanho populacional às condições ambientais variantes. Nesse contexto, Garland e Mills (1991) foram um dos precursores desta abordagem, utilizando o sistema Biolog. No entanto, Zak *et al*. (1994) foram os que introduziram o termo "Diversidade Funcional" e, dessa forma, propuseram o uso desse enfoque através do cálculo do índice de diversidade de Shannon (Magurran, 1998). A partir de então, muitas outras abordagens foram propostas, dentre elas o cálculo cinético de consumo das fontes de carbono (Lindstrom *et al*., 1998) e o cálculo da área da curva (Guckert *et al.*, 1996).

A avaliação da diversidade funcional através de padrões de utilização de substratos de carbono vem sendo aplicada, por cerca de 20 anos, por representar uma metodologia rápida, de fácil manipulação e que oferece um grande conjunto de informações. Neste sentido, diferentes trabalhos utilizando o sistema Biolog para acessar o potencial funcional de comunidade microbiana heterotrófica de amostras ambientais, vêm sendo conduzidos em ambientes aquáticos (Choi e Dobbs, 1999; Grover e Chrzanowski, 2000; Tiquia, 2010), solos (Goberna *et al,*. 2005; Epelde *et al.*, 2008; Sheehan *et al,*. 2008), banhados construídos (Weber *et al.*, 2008; Weber e Legge, 2009; Salomo *et al.*, 2009), lodo ativado (Guckert *et al*., 1996), entre outros.

O sistema Biolog é constituído por diferentes tipos de placas, que contém fontes de carbono específicas para acessar grupos de micro-organismos. Por exemplo, GN Microplates ^{TM}, para bactérias Gram-negativas; GP Microplate^{TM}, para Gram-positivas; e Ecoplate^{TM}. Este último é produzido especialmente para a análise de comunidades microbianas de amostras ambientais (Choi e Dobbs, 1999; Preston-Mafhan *et al.*, 2002).

2 OBJETIVOS GERAIS

Com vistas a investigar as informações a respeito da comunidade microbiana no contexto ecológico de seus atributos funcionais, este estudo enfocou a dinâmica metabólica da comunidade em uma lagoa subtropical. Para tanto, objetivos específicos foram definidos para guiar o presente estudo:

- acessar o potencial funcional da comunidade microbiana heterotrófica aquática, utilizando a abordagem de assinaturas metabólicas (*metabolic fingerprint*), através de padrões de utilização de fontes de carbono disponíveis;
- identificar a heterogeneidade funcional, considerando fatores intrínsecos da lagoa na determinação de dinâmicas temporais e espaciais;
- validar o método aplicado para fins de avaliação da dinâmica funcional microbiana em determinado contexto ecológico de um ambiente aquático.

3 MATERIAL E MÉTODOS

3.1 ÁREA DE ESTUDO

A Lagoa Mangueira (Fig. 1) é um sistema raso com profundidade máxima de seis metros e uma área aproximada de 820km^2. Este ecossistema é caracterizado por um sistema de a áreas úmidas em associação a lagos e lagoas, pertencendo ao Sistema Hidrológico do Taim, no Estado do Rio Grande do Sul. Localizado entre os municípios de Santa Vitória do Palmar e Rio Grande, está entre o Oceano Atlântico sul e a Lagoa Mirim, inserido na Unidade de Conservação da Estação Ecológica do Taim (ESEC-Taim), também fazendo parte da Bacia Hidrográfica da Lagoa Mirim.

Esta região é representativa de sistemas lacustres costeiros de água doce da região sul do Brasil, tipicamente complexos no que diz respeito à biodiversidade e dinâmicas de relações tróficas (Finkler-Ferreira *et al.*, 2007).

Fonte: Google Earth.

Fig 1. Área de estudo - Lagoa Mangueira, localizada no extremo sul do Rio Grande do Sul, Brasil.

O clima da região é subtropical (Cfa; Koöppen, 1936), o que torna essa região diferente de outras áreas alagáveis existentes no Brasil, devido à característica climática das quatro estações bem distribuídas ao longo do ano.

A Lagoa Mangueira sofre forte ação dos ventos, que predominam nos sentidos NE e SO (Fragoso Jr. *et al.*, 2008). Seu entorno é influenciado por diferentes características: na margem leste, por dunas, delimitando a zona marinha; na margem oeste, há variação no padrão de uso do solo, ocorrendo macrófitas aquáticas emergentes e zonas de cultivo de arroz irrigado (*Oriza* sp.); ao sul, ocorrência de macrófitas aquáticas submersas e ao norte ocorre o banhado do Taim, caracterizado por um maciço de vegetação aquática emergente. Este último tem grande potencial para exportação de matéria orgânica dissolvida e particulada para dentro da Lagoa (Motta Marques *et al.*,1997).

As características climáticas, edáficas e morfológicas da planície costeira, onde está inserido o SHT, fazem com que nessa região o cultivo de arroz irrigado esteja presente e, representando cerca de $110 m^3.s^{-1}$ de todo o consumo de água na época de primavera/verão (quando ocorre a drenagem para irrigação) (Tucci *et al*., 2002). Passando o período de irrigação, a água é devolvida para a Lagoa enriquecida por nutrientes e matéria orgânica (Motta Marques *et al*., 1997). Assim, gerando um potencial aumento do grau de trofia do sistema, capaz de promover alterações nas comunidades aquáticas em curto prazo.

3.2 DELINEAMENTO AMOSTRAL

As coletas foram realizadas durante as estações de verão, outono, inverno e primavera do ano de 2010. Nestes períodos foram coletadas simultaneamente amostras para análises das variáveis limnológicas e do perfil fisiológico em nível de comunidade microbiana.

3.2.1 Variáveis Limnológicas

3.2.1.1 Nutrientes

A análise de todos os nutrientes seguiu o método colorimétrico, utilizando o espectrofotômetro Varian Cary 1-E para a leitura da absorbância.

Formas nitrogenadas

Nitrogênio total – (APHA, 1999):

Consiste na oxidação dos compostos nitrogenados a nitrato, pelo aquecimento com solução de perssulfato de sódio alcalino sob pressão e posterior redução em coluna de cádmio. O nitrato é reduzido a nitrito com cádmio metálico. A determinação do nitrito ocorre pela diazotação com sulfanilamida, formando um composto colorido que é medido em espectofotômetro em 543nm em cubeta de 10mm.

Nitrato – (APHA, 1999):

Emprega a redução do nitrato em nitrito através da coluna de cádmio. A quantidade de nitrato da amostra é então determinada pela formação de um composto colorido através da reação com sulfanilamida. Esse composto é lido em espectofotômetro em 543nm.

N-Amoniacal – (Mackereth *et al.*, 1989):

O nitrogênio amoniacal reage com o fenol e o hipoclorito em solução alcalina, formando indofenol azul. O resultado de absorbância é proporcional à amônia presente na amostra e é medido em espectrofotômetro a 635nm.

Formas fosfatadas

Fósforo total - (Mackereth *et al.*, 1989):

Os íons ortofosfato e molibdato condensam em solução ácida. Então todo o fósforo se transforma em o ácido molibdofosfórico, que apresenta coloração azul, lido em espectrofotômetro a 880nm.

Fósforo Reativo ou Ortofosfato - (APHA, 1999):

Condensação ácida dos íons ortofosfato e molibdato, gerando ácido molibdofosfórico, de coloração azul, lido a 880nm em espectrofotômetro. A intensidade da cor azul é proporcional à quantidade de ortofosfato na amostra.

3.2.1.2 Série Carbono

As coletas das amostras para a análise de carbono foram feitas em vidros âmbar, calcinados a 450 ºC por uma hora, mantendo-se o bocal protegido da tampa de plástico por folha de alumínio. As amostras foram refrigeradas no escuro até a análise.

Os carbonos orgânicos considerados neste trabalho foram o Carbono Orgânico Total (TOC) e Carbono Orgânico Dissolvido (DOC).

Para quantificação dos carbonos, utilizou-se Analisador de Carbono Orgânico Total (Shimadzu 5000) para a leitura das amostras, no qual o IC (Carbono Inorgânico, DIC) e o TC (Carbono Total) foram lidos diretamente.

Carbono Orgânico Total (TOC)

Resulta da diferença entre Carbono Total (TC) e Carbono Inorgânico (IC), nas amostras brutas.

Carbono Orgânico Dissolvido (DOC)

Representa a amostra filtrada em filtros de fibra de vidro (Macherey-Nagel, 0,6 µm de retenção média), previamente calcinados a 450 ºC por uma hora. É o resultado da diferença entre TC e IC nas amostras filtradas.

3.2.1.3 Sólidos

A concentração de sólidos foi determinada por método gravimétrico, sendo discriminada a fração de sólidos suspensos totais (APHA, 1999):

Sólidos Suspensos Totais

Fração de sólidos da amostra, retidos em filtro 0,45µm e posteriormente secados em estufa a 105^{o}C. A diferença no peso inicial do filtro (antes de filtrar a amostra) e o peso final (após a filtração) representa os sólidos suspensos.

3.2.1.4 Temperatura da Água e pH

Medidos através da sonda Multiparameter Water Quality Sonde.

3.2.1.5 Turbidez

A turbidez foi medida através da sonda Multiparameter Water Quality Sonde. A análise consiste na comparação entre a intensidade de luz dispersada pela amostra sob condições definidas e a intensidade de luz dispersada por uma suspensão de referência padrão sob as mesmas condições.

3.2.1.6 Profundidade

Medida através de sistema sonar de mão.

3.2.1.7 Profundidade Secchi

Medido através do disco de secchi, indica uma aproximação da profundidade da zona eufótica.

3.2.1.8 Clorofila *a* – (Wetzel e Likens,1991)

A clorofila *a* se encontra na proporção 1:3 com a clorofila *b* e ambas são facilmente extraídas com solventes orgânicos. O método utilizado foi de extração com etanol e posterior leitura em espectrofotômetro a 664nm.

3.2.2 Perfil Fisiológico em Nível de Comunidade Microbiana

Para investigar os padrões da comunidade microbiana em relação à utilização potencial de fontes de carbono utilizou-se a abordagem de *metabolic fingerprints* através do uso de Biolog EcoPlate^{TM} (Biolog, Inc.,Hayward,CA). Biolog Ecoplate são placas compostas por 96 poços, divididos em três réplicas de 31 fontes de carbono com corante redox violeta tetrazolina e um controle negativo sem fonte de carbono. As 31 fontes de carbono podem ser agrupadas entre seis categorias de nutrientes: ácidos carboxílicos, aminas, aminoácidos, carboidratos, compostos fenólicos e polímeros Tab.1.

Tabela 1. Fontes de carbono separadas em seis categorias de nutrientes, conforme Choi e Dobbs (1999).

CÓDIGO	***AMINAS/AMIDAS***
G4	Feniletilamina
H4	Putrescina
	AMINOÁCIDOS
A4	L-Arginina
B4	L-Asparagina
C4	L-Fenilalanina
D4	L-Serina
E4	L-Treonina
F4	Ácido Glicil-L-glutâmico
	CARBOIDRATOS
H1	α-D-lactose
A2	β-Metil-D-glicosideo
G1	D-Celobiose
D2	D-Manitol
C2	I-Eritritol
G2	Glicose-1-fosfato
A3	Ácido D-Galactônico-ϒ-lactone
E2	N-Acetil-D-glicosamina
H2	D,L-α-glicerol fosfato
B2	D-Xylose
	ÁCIDO ACÉTICO CARBOXÍLICO
G3	Ácido α-ketobutírico
B3	D- Ácido Galacturônico
F2	D-Ácido Glicosamínico
E3	Ácido ϒ-Hydroxybutírico
H3	D-Ácido Málico
B1	Ácido Pirúvico metil éster
F3	Ácido Itacônico
	POLÍMEROS
E1	α-Ciclodextrina
F1	Glicogênio
C1	Tween 40
D1	Tween 80
	COMPOSTOS FENÓLICOS
C3	2-Hidroxi Ácido benzóico
D3	4-Hidroxi Ácido benzóico

A análise de utilização das fontes de carbono baseia-se na produção de cor lilás originada da redução da violeta tetrazolina usada como um indicador de respiração de uma única fonte de carbono (Garland e Mills,1991). Portanto, qualquer desenvolvimento de cor no controle negativo indica a utilização de fontes de carbono inerentes à amostra inoculada (Choi e Dobbs, 1999).

Neste trabalho, utilizaram-se amostras de água pré-filtradas em malha de 20µm para eliminar detritos e a influência da respiração do fitoplâncton. Após a coleta, as amostras foram refrigeradas até a incubação.

O procedimento de incubação foi realizado em câmara de fluxo laminar, utilizando pipeta multicanal (8 canais). Incubou-se 150µL de cada amostra diretamente em cada um dos 96 poços. As Ecoplacas foram mantidas em uma temperatura média de 25^{o}C. Manter a uma mesma temperatura de incubação previne variações espaciais e temporais na utilização do substrato, que seriam influenciadas pela variabilidade de temperatura (Christian e Lind, 2006). O consumo das fontes de carbono foi acessado pela leitura de densidade óptica (OD) a 590nm, através do leitor de placas SpectraMax 5.0 (Molecular Devices). A leitura da OD foi realizada imediatamente após a incubação (t=0h), e a cada 24h por 12 dias. As leituras foram realizadas por 12 dias para assegurar que a saturação da taxa de utilização fosse alcançada em cada amostra, conforme Salomo (2009). Todos os procedimentos foram realizados em condições estéreis.

3.2.2.1 Análise do desenvolvimento de cor

Os dados de absorbância bruta de cada poço em cada placa foram corrigidos descontando-se os valores do t=0, gerando novos valores de absorbância. Esse desconto evita a absorbância intrínseca do carbono inerente à amostra (Tiquia, 2010). A seguir, calculou-se a taxa global de desenvolvimento de cor, ou seja, *Average Well Color*

Development (AWCD), seguindo o cálculo proposto por Garland e Mills (1991), que corresponde ao somatório dos valores de absorbância das 31 fontes, subtraído os controles negativos (1). Ainda, valores negativos foram considerados zero.

$$AWCD = \sum \frac{(OD_{substrato} - OD_{controle})}{31} \quad (1)$$

A média de AWCD das três réplicas foi plotada num gráfico ao longo do tempo para cada amostra, permitindo, desta forma, acompanhar graficamente as diferentes fases da curva (lag, taxa máxima de desenvolvimento de cor e a assíntota) e a visualização da saturação média da taxa de utilização dos substratos.

Após a tabulação dos dados e do cálculo da AWCD foram aplicadas diferentes abordagens. As análises dos dados gerados pelo Biolog Ecoplate incluíram uma abordagem cinética (Lindstrom *et al*., 1998), calculada a partir da AWCD, a qual permitiu uma avaliação do metabolismo geral desenvolvido pela comunidade microbiana presente nas placas; e o método da integração da curva (Gukert *et al*., 1996), que representou o cálculo da área sob a curva de cada uma das 31 fontes de carbono.

A abordagem cinética consiste no ajuste da equação de crescimento logístico dependente da densidade à curva de desenvolvimento da cor pelo tempo. Nessa abordagem, dois parâmetros cinéticos foram calculados, por serem independes da densidade do inóculo (Lindstrom *et al*., 1998). O parâmetro *K*, que corresponde à assíntota da curva; e *r*, que corresponde à taxa exponencial de mudança da AWCD. A abordagem da integração da curva foi escolhida para acessar o metabolismo de cada uma das 31 fontes de carbono, pois esse método correlaciona-se aos parâmetros cinéticos (Garland *et al*., 2001) e resume num único valor as diferentes fases da curva de desenvolvimento de cor (Gukert *et al*., 1996). O cálculo da área sob a curva é uma aproximação da área do trapezóide (2).

$$Área\ do\ Trapezóide = \sum_{i=1}^{n} \frac{v_i + v_{i-1}}{2} \times (t_i - t_{i-1}) \quad (2)$$

onde v_i é a densidade óptica no tempo t_i. Este método permite uma análise mais robusta do padrão metabólico da comunidade microbiana, uma vez que o valor gerado resume as diferenças nas várias fases ao longo do tempo de incubação (Guckert *et al.*, 1996).

Outra abordagem utilizada neste estudo foi a investigação da diversidade de substratos utilizados em cada placa. Esse método seguiu o proposto por Zak *et al*. (1994) e Weber *et al*. (2008). O Índice de Shannon foi calculado seguindo a fórmula: $H = -\sum p_i \ln(p_i)$, onde H é a diversidade de substrato, *pi* é a taxa da atividade de um substrato em particular, dividido pela soma da atividade de todos os substratos,

4 CAPÍTULO 1

4.1 APRESENTAÇÃO

O estudo apresentado a seguir trata de aspectos relacionados à funcionalidade da comunidade microbiana aquática. Investiga fatores que estejam favorecendo possíveis padrões funcionais relacionados às características ecológicas da Lagoa Mangueira.

Functional heterogeneity of heterotrophic microbial communities in a subtropical sallow lake

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Abstract

Communities vary in time and space as a result of their interactions with the environment and other organisms. Aquatic microbial communities are important components in the aquatic metabolism, recycling nutrients by remineralization, and transferring biomass through the microbial food web. Functional microbial dynamics in lakes is an important tool for understanding these systems, since the microbial composition and function may reflect the overall condition of the lake. Therefore, we used the approach of metabolic fingerprint of microbial communities in shallow lakes to assess the functional attributes of the heterotrophic microbial community through consumption patterns of known carbon sources using Biolog Ecoplates^{TM} and to recognize the existence of temporal and spatial patterns of utilization preference in these substrates. The study was conducted in a coastal and large subtropical shallow lake, located in southern Brazil. Different kinds of data were generated for functional diversity. During summer and autumn all 31 substrates were used, while winter and spring only skipped the carboxylic acid y-hydroxybutyric. Temporal and spatial patterns were evident for both limnological and functional potential data. Such patterns were drove mainly by nutrient dynamics and productivity components. Kinetic data showed positive correlation with turbidity, total suspended solids and chl*a* and negative correlation with water transparency. In the analysis of indicator substrates, different carbon sources presented relevance of consumption for different seasons and for different sampling sites in the Lake. Redundancy analysis (RDA) showed that the majority of substrates potentially indicators were determined by one or more limnological variables, confirming that the temporal and spatial pattern of preference and use of carbon sources for the study in question was the result of the interaction of community with the characteristics of the environment. Carbon source utilization approach, in the present study, was a good indicator of functional dynamics in this aquatic ecosystem.

Keywords: *microbial functional diversity, shallow lake, temporal and spatial pattern.*

1. Introduction

A common feature in plankton distribution in aquatic system is the patchiness, where plankton occurs on a hierarchical continuum of scales in these systems (Pinel-Alloul *et al*., 1995). This patchiness is fundamental to population dynamic, community organization, and spatiality (Mehner *et al*., 2005). Lakes microbial communities have great potential for temporal and spatial changes within and between habitats (Garland, 1997; Yannarell and Triplett, 2004), and respond rapidly to environmental changes (Allan, 1995), as a result of their interactions with other organisms, and the conditions and resources availability in the habitat. Also represent a powerful tool for understanding community dynamics in ecological contexts (Garland, 1997).

Functional heterogeneity links ecological interactions between organisms and environmental process, and operate hierarchically over different scales of the environment and different level of biological organization (Kolasa and Rollo, 1991). The bacterioplankton assembly may functionally and compositionally reflect the whole lake conditions (Haig-They *et al.,* 2010). Still, bacterioplankton dynamic in lakes is strongly influenced by a bottom-up regulation (Thomaz, 1999), where nutrients viability and physical-chemistry water characteristic can limit bacterial function and composition.

Aquatic microbial population can play an important role in degrading organic matter and transferring nutrient through the food web via microbial loop (Azam, *et al*., 1983). By this way, they are liable to transform the net primary production (Tammert *et al*., 2002), releasing nutrients directly to surrounded water or being grazing by their predators and then indirectly transferring nutrients.

Coastal shallow lakes are mainly driven by littoral zones and aquatic plants, rather than phytoplankton, dominate the primary production (Moss, 1998) which presents high productivity and where the accumulation of organic matter in the bottom of the lake exceeds its consumption (Danovaro and Pusceddu, 2007). There is a tendency to regard such lakes as exceptions but this is to underestimate their extent and importance (Moss, 1998).

Heterotrophic microbial community functionality and diversity can play a role in aquatic ecosystems metabolism, related to their capabilities to incorporate carbon derived from primary production and both transferring biomass through microbial food wed, becoming a link in the food web via microbial loop, and recycling nutrients by remineralization (Azam *et al.*, 1983; Cotner and Biddanda, 2002). Considering such important function, it would be useful to have an applicable technique for understanding microbial community functionality heterogeneity in aquatic systems.

Patterns of carbon-source utilization has been used as an useful and rapid method to characterize heterotrophic microbial communities in various environments, like freshwater (Garland and Mills, 1991; Choi and Dobbs, 1999; Grover and Chrzanowski, 2000; Leflaive, *et al.,* 2008; Tiquia, 2010), soils (Goberna *et al.,* 2005; Epelde *et al.*, 2008; Sheehan *et al.,* 2008), constructed wetlands (Weber *et al.*, 2008; Weber and Legge, 2009; Salomo *et al.,* 2009), activated sludge (Guckert *et al*., 1996) and rhizosphere (Garland, 1995). However, most studies involving microbial communities of freshwater have been conducted in temperate lakes (e.g. Choi and Dobbs, 1999; Grover and Chrzanowski, 2000; Leflaive, *et al.,* 2008), whereas subtropical shallow lakes have only received negligible attention, mainly for composition and diversity studies (e.g. Haig-They, *et al*., 2010; Rodrigues *et al*., 2011; Canterle *et al.*, unpublished data**).**

So far, analysis of functional diversity, looking for temporal and spatial patterns driven by water characteristics still remains poorly elucidated. Therefore, the aim of this study was to determine patterns on temporal and spatial scales derived from water characteristics and metabolic diversity of the heterotrophic microbial community assessed by Biolog Ecoplate^{TM}. Also verify if patterns of substrate utilization were being driven by water characteristic which is likely determining the short-terms in a large, coastal, shallow lake.

Our hypothesis is that heterotrophic microbial function heterogeneity could be assessed by Community-Level Physiological Profile (CLPP) approach and this assay allows verify short-terms patterns in the community, driven by environmental characteristics of the lake.

2. Materials and Methods

2.1. Sampling Site

The study area was Lake Mangueira (Fig.1), a large and coastal shallow lake (820km^2) located in Rio Grande do Sul, southern Brazil (~30^{o}31'22"S 53^{o}07'48"W). The Lake is surrounded by fixed dunes in the Atlantic Ocean side, by wetlands in the north and south and by rice field in the west side. Since the study proposal was to investigate temporal and spatial variations, the sampling of surface water was carried out at four different dates during 2010: summer (February), autumn (May), winter (August) and spring (November); across all Lake, in south, center and north (extreme zones); and in west side, pelagic zone and east side (littoral zones).

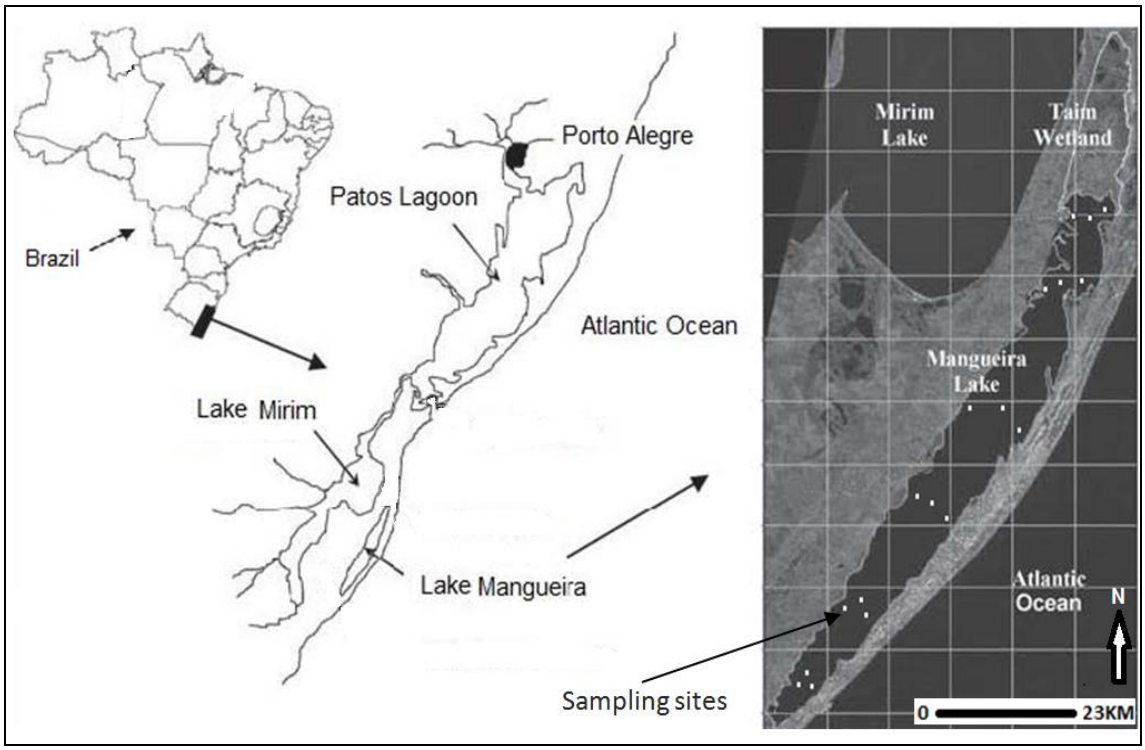

Fig. 1 Lake Mangueira, Rio Grande do Sul, southern Brazil. White dots inside the lake represent the sampling sites.

2.2. Variables Analyzed

2.2.1. Limnological Variables

The Turbidity (Turb), pH and water temperature were measured with a Multiparameter Water Quality Sonde. Depth was estimated with handheld sonar system and water transparency with secchi disk. Total Suspensed Solids (TSS) were assessed gravimetrically by water evaporation in porcelain dishes (APHA 1999). Nutrients were determined through colorimetric methods: Total Nitrogen (TN), Nitrate (NO_3^-), and Soluble Reactive Phosphorus (PO_4^{-3}) following APHA (1999). Analysis of ammonium (NH_4^-) and Total Phosphorus (TP) followed Mackereth *et al*. (1989). Chlorophyll a (Chla) was quantified after ethanol extraction (Wetzel and Likens, 1991). Carbon series were determined in Carbon Analyzer (Shimadzu Vcph 5000): Total Organic Carbon

(TOC) was the unfiltered sample and the Dissolved Organic Carbon (DOC) was accounted as the fraction that passed through a 450 ºC pre-combusted glass fiber filter (Macherey-Nagel GF6 - 0,6µm average mesh size).

2.2.2.Assessment of substrate utilization potential

Biolog Ecoplate™ (Biolog, Inc. Hayward, CA) was used to assess the heterotrophic microbial community present in the water samples, determined by the "metabolic fingerprint", based on the carbon source they consume. For this analysis, the water lake was collected through 20µm mesh size sieve in 50 ml of amber glass bottle to avoid phytoplankton and detritus interference and then stored in the dark under refrigeration until analysis. Biolog Ecoplates™ was specially produced for microbial community's analysis of environmental samples (Insam, 1997; Choi and Dobbs, 1999; Preston-Mafhan *et al.*, 2002; Salomo, 2009). It comprises three replicates of 31 ecologically relevant carbon sources and one control. In each well, redox dye (tetrazolium dye) is added and the metabolism of the substrate in a particular wells results in formazan production, which turns purple, when the carbon source is used by the microorganism into the sample.

In each well of Biolog Ecoplate, 150µl of water Lake were directly inoculated and incubated at approximately 25^{o}C. According to Christian and Lind (2006), pre-selected incubation temperature prevents spatial and temporal variation in substrate utilization due to variability in incubation temperature. For each water sample, three replicates were prepared. The metabolism of the substrate was assessed by measuring the optical density (OD) at 590nm with an automated plate reader (SpectraMax 5.0, Molecular Devices). The OD was read immediately after incubation (t=0h) and every 24h during 12 days, until OD values showed a turnover saturation. The measurement was practiced

for 12 days to ensure that a saturation of the utilization rate in all samples was reached (Salomo, 2009).

The analyses of Biolog Ecoplate data included the average well color development (AWCD) method (Garland and Mills, 1991) where a kinetic approach (Lindstrom *et al.*, 1998) of the AWCD was used to evaluate the overall metabolism. Also, the curve integration (CI) method (Gukert *et al*., 1996) was used to evaluate the proportional substrate utilization efficiency (PSUE) (Pietikäinen et al., 2000) of the 31 carbon source. For all analysis, data from the triplicate substrate were averaged.

Primarily, for each reading time, raw absorbance data were corrected by subtracting the absorbance values from the first reading (t=0h). Using the corrected data the average well colour development (AWCD) was calculated according to Garland and Mills (1991), i.e., AWCD=Σ(C-R)/n where C is corrected color production, R is the absorbance values of the plate's control well, and n is the number of substrate (n=31 for Ecoplates). The kinetic approach was used to overcome possible effects of inoculums density and time (Lindstrom *et al.*, 1998; Preston-Mafham *et al.*, 2002). Thus fitting the kinetic model to the color development data, removes the effect of the lag period and the actual rate of color change was determined. Two kinetic parameter (*K* and *r*) were used in this study because do not vary with inoculums density (Lindstrom *et al.*, 1998). The kinetic parameters were estimated fitting a density-dependent logistic growth equation based on a sigmoid curve (Lindstrom *et al.*, 1998), using the software LabFit (Silva and Silva, 2004):

$$Y = OD_{590nm} = \frac{K}{\left(1+e^{-r(t-s)}\right)}$$

where *K* represents the asymptote (*y*=*k*) and *r* determines the exponential rate of OD change, and *t* is the time following incubation of the microplate. The *parameter s* is the time to the midpoint of the exponential portion of the curve (when *y*=*k*/2). This last parameter was not measured because according to Lindstrom *et al.* (1998) is influenced by inoculums density, i.e.; the value of *s* decrease with increasing inoculums density.

According to Zak *et al.* (1994), and following Weber *et al*. (2008) the substrate diversity was estimated using Shannon's diversity index ($H' = -\sum p_i \ln(p_i)$) (Magurran, 1988), where H' is the substrate diversity, *pi* the ratio of the activity of a particular substrate to the sum of activities of all substrate activity. In this study, we calculated the diversity of all time of incubation when evaluating the temporal variation. For spatial variation, the time point at 192h and at final incubation (288h) was investigated.

The CI was calculated according to Guckert *et al.* (1996) method, which is a trapezoidal approximation of each of 31 carbon source curves. This approach incorporate additional information from the absorbance versus the time incubation (lag phases, rates of color development and maximum absorbance) into a single number (Guckert *et al*., 1996) and is correlated with the kinetic parameters (Garland et al., 2001). The single value obtained was transformed in proportional substrate utilization efficiency (PSUE) to avoid effects of inoculums density (Pietikäinen *et al*., 2000).

2.3. Statistical Analysis

Data matrices were matched in time/space (n=72) and only space (n=18) intervals to explore information about temporal and spatial variation. The limnological data was log(x+1) transformed and a Principal Component Analysis (PCA) was performed. Then *one-way* MANOVA (to evaluate temporal pattern) and *two-way* MANOVA (looking

spatial patterns) were used to test significance differences. Analysis of variance (ANOVA) was calculated between each limnological data looking for what varied in each season and in each site. When analysis presented more than two groups (i.e., summer, autumn, winter and spring for temporality, and extremes and littoral zones for spatiality) contrasts were used to find which groups were different from each other. Contrasts were as well evaluated by randomization testing (Pillar and Orlóci, 1996).

ANOVA`s were also performed for CLPP data, using *k* and *r* kinetic parameter and substrate diversity H', for temporal and spatial changes of the overall metabolism. When looking for temporal and spatial variation of PSUE data, a MANOVA was carried out. Contrasts analysis was also used. Further a detrended correspondence analysis (DCA) using CI data was performed to establish the lengths of first gradient, which were less than 3.0 standard deviation units, indicating that data should be subject to linear ordination methods (PCA, RDA) (Ramette, 2007). Thus, to analyze the spatiality within each season, a PCA ordination was made with PSUE data of the 31 carbon source, where a cross-product matrix was based on variance/covariance (Dufrêne and Legendre, 1997).

Furthermore, an indicator species analysis was performed following Dufrêne and Legendre (1997) method which derives indicator species from any hierarchical or non-hierarchical classification of the objects (sampling sites), based only on within-species abundance and occurrence comparisons; its value is not affected by the abundances of other species. The indicator values vary from 0%, for substrates that have similar occurrence and abundance in all season, to 100% for substrates that were restricted utilized in only one season (Legendre and Legendre, 1998). The significance of the indicator value of each species was assessed by a randomization procedure. In the context of Biolog^{TM} the term indicator species analysis was replaced by "indicator

substrate analysis" as we are dealing with substrate utilization patterns as opposed to direct counts. This analysis allowed finding which substrate was more associated in each group of time and space.

Looking for relationships between the consumption parameters (kinetic profiles and efficiency of consumption) and the environmental factors, Pearson's product-moment correlations were carried out. Furthermore, Redundancy Analysis (RDA) was performed to statistically evaluate the main environmental factors shaping the utilization of available carbon source by the bacterial assemblage. According to Rao (1964), this method can be considered as an extension of PCA, in which the main axes are constrained to be linear combinations of the environmental variables. For that, multiple linear regressions are used to 'explain' variation between independent (environmental) and dependent (carbon utilization) variables. The data from significant ($P<0.05$) Pearson's product-moment correlations were used in the RDA. This was taking account to avoid input of noisy or irrelevant explanatory variable (McCune, 1997), that may lead to misleading interpretations (Legendre and Legendre, 1998).

The analyses of variance were based on Euclidian distance (Legendre and Legendre, 1998), used as a measured of resemblance among sites for both limnological and CLPP data. For PCA and RDA ordination and indicator substrate analysis, PCord 6.0 software (McCune and Mefford, 1999) was used. For ANOVA and MANOVA, Multiv v.2.4.2 (Pillar, 2006) was used.

3. Results

3.1. Limnological Scenario

The PCA ordination explained 80.75% of data variability on the two first axis (Fig.2), and both axis contribute significantly ($P<0.001$) to the variation of the data.

Axis 1 is completely separating summer from the other seasons. The parameters explaining the variation along axis 1 were Chl*a* (r=0.84), TSS (r=0.78), pH (r=0.76), depth (r=0.72), TP (r=0.59), TN (r=0.53), TOC (r=0.37) and DOC (r=0.35). Spring, winter and autumn were being slightly separated across axis 2.

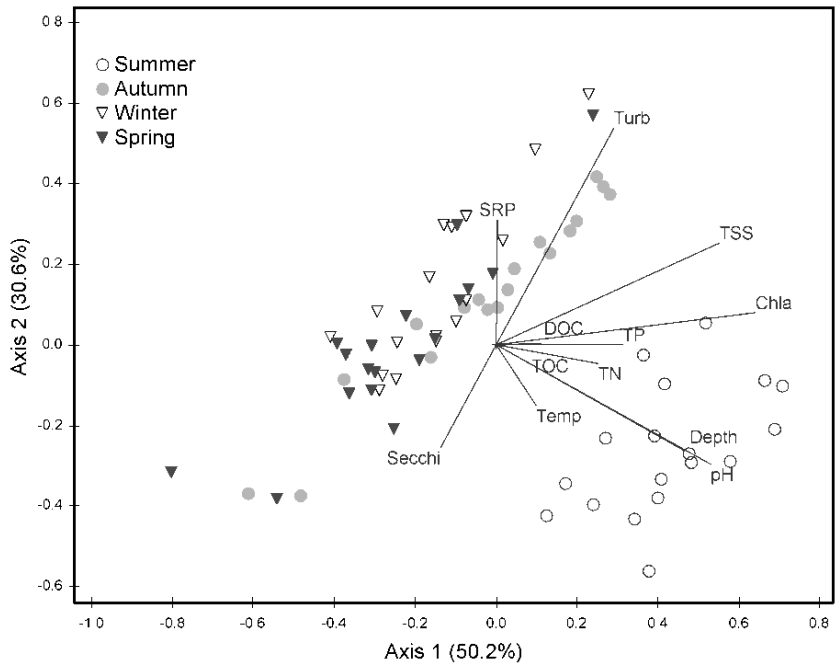

Fig. 2 PCA biplot diagram of limnological data collected during summer, autumn, winter and spring at Lake Mangueira.

Along axis 2, turbidity (r=0.78), SRP (r=0.59), water transparency (secchi) (r=-0.53) were the main factors responsible for the ordination of the samples. Turbidity was more related to autumn and was negatively correlated to transparency which was more related to spring and winter. Temperature showed some separation between summer and the others season along axis 2. Ammonium and nitrate, did not showed significant (P>0.05) correlation (r>0.24) between axis. Thus, surface water characteristics differed on temporal scaling (P=0.001). All season differed at least in six characteristics (Table 1).

Table 1. Limnological data (mean and standard deviation) of the water samples from Lake Mangueira during summer, autumn, winter and spring.

Seasons		Turbidity (NTU)	TSS ($mg.l^{-1}$)	TP ($mg.l^{-1}$)	SRP ($mg.l^{-1}$)	TN ($mg.l^{-1}$)	NH_4^+ ($mg.l^{-1}$)	NO_3^- ($mg.l^{-1}$)	Chl*a* ($ug.l^{-1}$)	TOC ($mg.l^{-1}$)	DOC ($mg.l^{-1}$)	Depth (m)	Secchi (m)	pH	Temperature (°C)
Summer (March 2010)	Mean	4.9	14.50	0.04	0.01	0.48	0.05	0.07	6.20	16.67	15.59	2.92	1.16	8.54	22.75
	SD	2.11	4.17	0.007	0.002	0.120	0.11	0.05	2.01	0.78	1.29	1.53	0.26	0.12	1.05
Autumn (May 2010)	Mean	6.33	14.31	0.023	0.013	0.434	0.03	0.06	3.79	17.41	16.29	2.74	0.94	8.13	17.79
	SD	2.81	6.57	0.007	0.005	0.074	0.02	0.01	1.58	0.68	1.11	1.52	0.31	0.05	0.34
Winter (August 2010)	Mean	5.61	10.58	0.028	0.012	0.257	0.04	0.11	4.63	16.26	15.22	3.09	1.58	8.04	11.68
	SD	2.74	4.86	0.016	0.002	0.036	0.02	0.02	1.72	1.10	0.92	1.63	0.64	0.09	0.44
Spring (November 2010)	Mean	4.08	10.06	0.029	0.016	0.216	0.04	0.13	2.95	14.00	13.05	3.30	1.08	8.01	22.16
	SD	3.07	5.48	0.008	0.004	0.133	0.02	0.09	1.09	0.58	0.78	1.64	0.25	0.10	0.66
ANOVA *P-values*		0.09^{ns}	0.029 *	0.001 **	0.001 **	0.001 **	0.931 ^{ns}	0.001 **	0.001 **	0.001 **	0.001 **	0.759 ^{ns}	0.001 **	0.001 **	0.001 **
summer vs. autumn		ns	ns	0.001 **	0.051 /ns	ns	ns	ns	0.003 **	0.009 **	ns	ns	0.034*	0.001 **	0.001 **
summer vs. winter		ns	0.016 *	0.005 **	0.021 *	0.001 **	ns	0.001 **	0.014 *	ns	ns	ns	0.02 *	0.001 **	0.001 **
summer vs. spring		ns	0.015 *	0.001 **	0.001 **	0.001 **	ns	0.012 *	0.001 **	0.001 **	0.001 **	ns	ns	0.001 **	0.051 /ns
autumn vs. winter		ns	ns	ns	ns	0.001 **	ns	0.001 **	ns	0.003 **	0.006 **	ns	0.001 **	0.003 **	0.001 **
autumn vs. spring		ns	0.035 *	0.017 *	ns	0.001 **	ns	0.001**	ns	0.001 **	0.001 **	ns	ns	0.001 **	0.001 **
winter vs. spring		ns	ns	ns	0.001 *	ns	ns	ns	0.001 **	0.001 **	0.001 **	ns	0.009 **	ns	0.001 **

NTU, nephelometric turbidity units; TSS, total suspended solids; TP, total phosphorus; SRP, soluble reactive phosphorus; TN, total Nitrogen; Chl *a*, chlorophyll *a;* TOC, total organic carbon; DOC, dissolved organic carbon; vs., versus. *ANOVA* *P≤0.05; **P≤0.01; ns= not significant.

Summer presented higher average values of TSS and TN comparing to winter, and spring. TP, pH and Chl*a* were higher in summer than autumn, winter and spring. Water temperature was very similar for both summer and spring, being higher than autumn and winter. SRP had higher average values during spring. TOC and DOC were higher in autumn. Winter did not presented higher average values than the others. Turbidity, ammonium, and depth did not vary significantly throughout the season.

Spatial variation was also observed within each season in the PCA ordination diagram for environmental variables. In Fig. 3 is presented the variation across north, center and south (extremes) during summer, winter and spring. As well the diagram showed variation along the littoral zones during winter and spring. On Table 2 this variation was confirmed by *two-way* ANOVA. Comparing extremes zone and littoral zones, summer presented variation only between south and center of the lake. During autumn, north was different from south and from center of the lake. In autumn and in summer did not occurred significant differences between littoral zones, whereas winter presented completely variation across spaces. Spring showed south being different from center and from north. Spring also had variation between west side, pelagic zone and east side. The variation of each limnological parameter was performed within each season and the results are present in Appendix 1. Ammonium, nitrate, DOC and TOC did not presented significant spatial variation across any season. Depth was significantly ($P<0.05$) higher in the pelagic zone than the west side for all seasons. Center of the lake during summer presented higher average values for SRP and Chl*a*. North of the lake had significantly ($P<0.05$) higher values of TN, nitrate and pH comparing to the center of the lake during autumn.

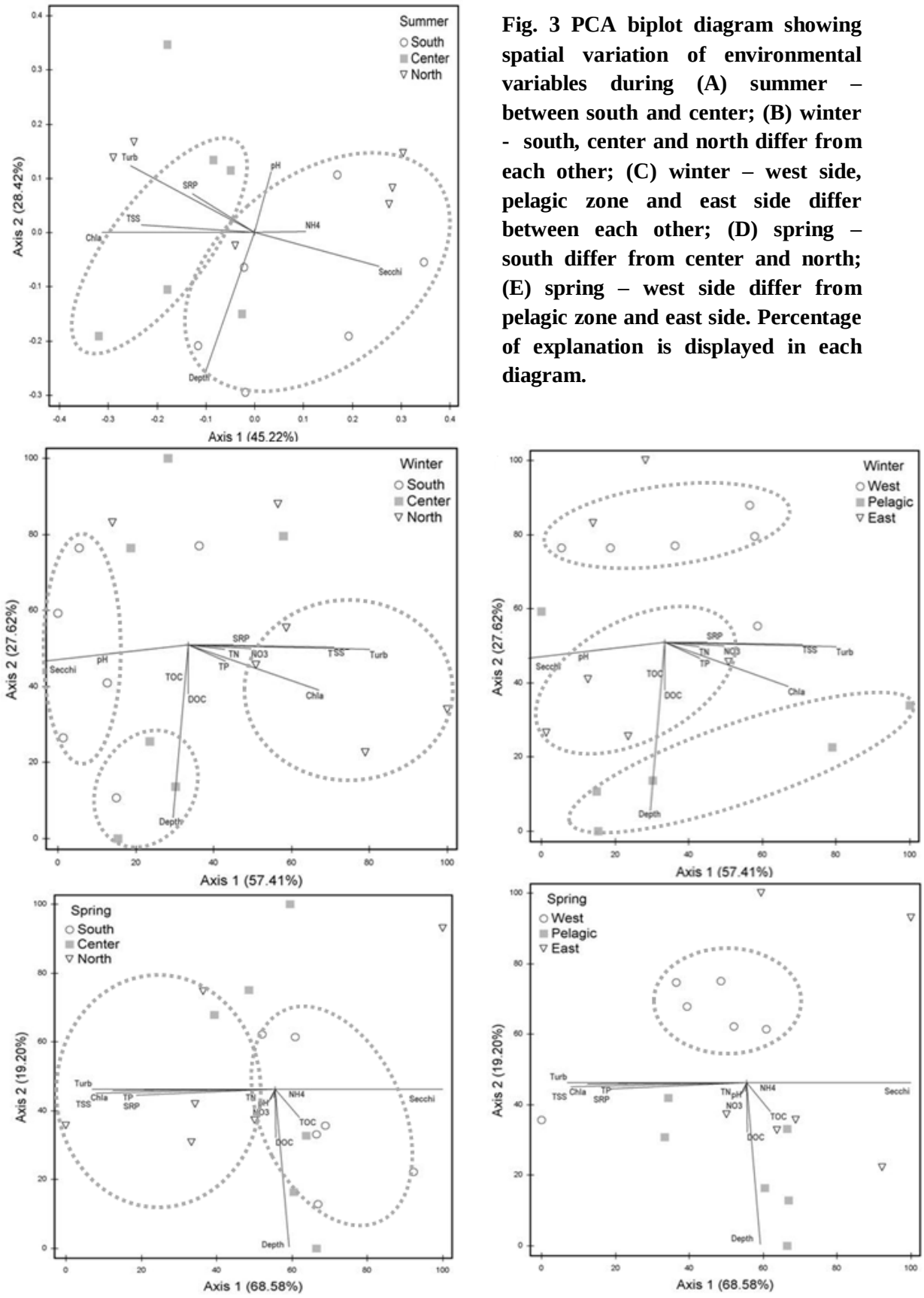

Fig. 3 PCA biplot diagram showing spatial variation of environmental variables during (A) summer – between south and center; (B) winter - south, center and north differ from each other; (C) winter – west side, pelagic zone and east side differ between each other; (D) spring – south differ from center and north; (E) spring – west side differ from pelagic zone and east side. Percentage of explanation is displayed in each diagram.

Table 2. Spatial variation of the limnological data within each season at Lake Mangueria.

Space interaction	Summer	Autumn	Winter	Spring
	P-Values	*P-Values*	*P-Values*	*P-Values*
Extreme	0.011 *	0.002 **	0.001 ***	0.002 **
South X Center	0.025 *	ns	0.024 *	0.006 **
South X North	ns	0.048 *	0.015 *	0.018 *
Center X North	ns	0.007 **	0.005 **	ns
Littoral	0.141 ns	0.107 ns	0.001 ***	0.005 **
West X Pelagic	ns	ns	0.013 *	0.011 *
West X East	ns	ns	0.009 **	0.029 *
Pelagic X East	ns	ns	0.013 *	ns
ext x littoral	ns	ns	ns	ns

Manova *two-way* (n=18): *P≤0.05; **P≤0.01; ***P≤0.001; ns, not significant.

3.2. Community – level physiological profile (CLPP)

3.2.1. Temporal Patterns

Kinetic Profile

The average well color development (AWCD) represented the typical sigmoid curve course over the time incubation, for all samples during the four seasons. These AWCD curves (Fig.4) were the basis for further interpretation of kinetics profiles of the community.

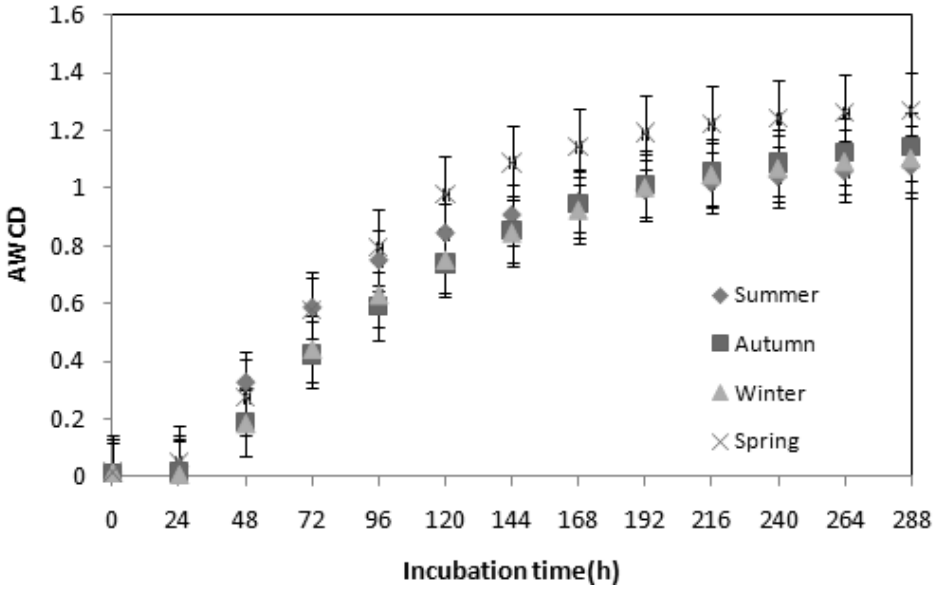

Fig. 4 Time course change of AWCD of each season. Mean and standard deviation are displayed (n=18).

The kinetic parameters obtained from fitting the AWCD curves to the density-dependent logistic growth equation are indicated in Table 3. The maximum degree of substrate utilization represented by *K* parameter showed significantly (P<0.01) higher during spring than summer, autumn and winter which had no significant differences. On the other hand, the maximum exponential rate (*r* value) was equal for summer and spring being significantly higher than autumn and winter which had also no significant differences.

Table 3. Kinetic parameter, *K* and *r* from fitting the AWCD curve to the density-dependent logistic growth equation.

Season	*K*		*r*	
	mean	SD	mean	SD
Summer	1.02	0.024 [a]	-0.04	0.006 [a]
Autumn	1.08	0.026 [a]	-0.03	0.003 [b]
Winter	1.07	0.028 [a]	-0.03	0.004 [b]
Spring	1.23	0.019 [b]	-0.04	0.003 [a]

Signal of minus, population is decreasing. Different letter are significantly different. Mean values (n=18). ANOVA P≤0.01.

Shannon Diversity Index (H') of substrate utilization

Fig. 5 show significant differences of Shannon diversity of substrate utilization during spring, comparing it to summer, autumn and winter. In spring, H' was higher in average for all incubation time compared to other seasons. For the first 48h (lag phase) all H' had no differences, but as time passed by, during exponential phase (from 72h to 216h) spring showed higher H'. During this phase (approximately from 168h to 216h) samples from winter presented similarities with samples from spring and also with summer and autumn. When time of incubation followed the asymptote (216h – 288h), spring back the only one with significantly higher value of substrate diversity.

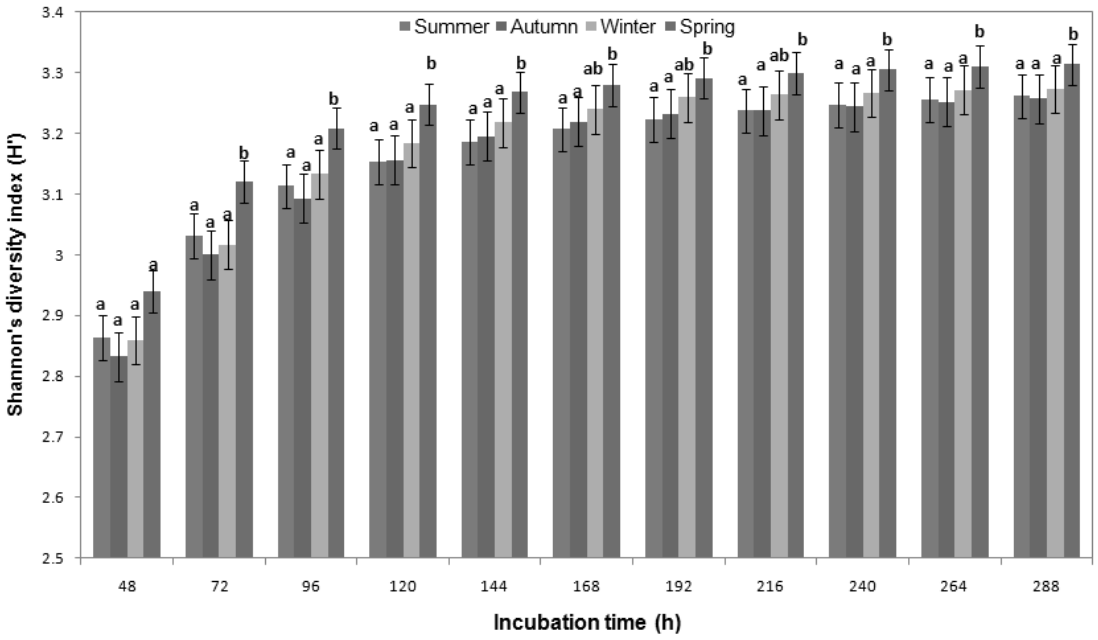

Fig. 5 Shannon diversity index (H') of carbon substrate diversity derived from CLPP`s analysis of sample from summer, autumn, winter and spring, for each incubation time. Means followed by same letter do not differ. Different letters show significant difference. ANOVA, P≤0.05. Mean values (n=18).

Substrate Utilization of Heterotrophic Metabolism

Microbial communities sampled during summer and autumn were able to catabolism all of the 31 carbon sources. In general, winter and spring were only able to consume 30 substrates. The carboxylic acid, y-Hidroxybutyric acid was not labeled for all samples both in winter and spring.

All seasons differed between each other according to substrate utilization pattern (*one-way* MANOVA, P=0.001). The indicator substrate analysis significantly ($P \leq 0.05$) identified a total of 11 substrates in association with the seasons (Table 4). In this study, indicator values ranged from 27.5% to 46.4%. In this way, four substrates with high efficiency of utilization were associated with summer, belonging to three guilds of substrate (amines, amino acids and carboxylic acids). The carboxylic acid, *y*-Hydroxybutyric acid, presented the highest percentage of perfect indication (46.4%). Autumn presented three substrates significantly associated to this season. These substrates belong to two guilds of substrates (polymers and carbohydrates) and presented similar percentage of perfect indication ranging from 27.5% to 30.4%. Only two substrates of carbohydrate were associated with winter: I-Erythritol that presented higher percentage of indication (40.2%) and Mannitol with low, but significant indicator value (28.3%). Spring also presented only two substrates with similar percentages of indication: Itaconic acid with 31.4% and α-Ketobutyric acid with 35%, both carboxylic acids. Phenolic compounds did not presented association with any season.

Table 4. Indicator substrates analysis of overall season.

Carbon Sources		Summer		Autumn		Winter		Spring	
	Code	% of perfect indication	P-*values*	% of perfect indication	P-*values*	% of perfect indication	P-*values*	% of perfect indication	P-*values*
Amines/amides									
Putrescine	H4	29.3	0.021*						
Amino acids									
L-Arginine	A4	28.9	0.0065**						
L-Serine	D4	28.6	0.0038**						
Carbohydrates									
D-Cellobiose	G1			27.5	0.046*				
D-Mannitol	D2					28.3	0.0007***		
I-Erythritol	C2					40.2	0.0004***		
Carboxylic & acetic ac.									
α-ketobutyric acid	G3							35	0.0015**
Υ-Hydroxybutyric acid	E3	46.4	0.0036**						
Itaconic acid	F3							31.4	0.0009***
Polymers									
Glycogen	F1			30.4	0.0071**				
Tween 80	D1			28.1	0.006**				

Indicator substrate analysis *P≤0.05; **P≤0.01;***P≤0.001

3.2.2. Spatial Patterns

The spatial variation of functional parameters is presented here in two approaches: firstly an overview of spatiality, including data from all season (n=72) in which a MANOVA and an indicator substrate analysis were performed; secondly, the spatiality within each season (n=18). In Table 5 is summarized the mainly results of the multivariate analysis (PCA, indicator substrate analyses and *two-way* MANOVA) for autumn, winter, and spring.

Spatiality

The overall utilization of the available carbon sources showed spatial heterogeneity in Lake Mangueira. The littoral differed from pelagic zone (MANOVA, P=0.021) and extremes zones, including north, center and south of the lake, also were heterogenic (MANOVA,

P=0.001). The indicator substrate analysis revealed that mainly substrates were associated with samples from north comparing to other sites. Thus four substrates were significantly (P<0.05) associated with the north side: a carbohydrate, D,L-α-glycerol phosphate (46.7% indicator value); an amino acid, L-threonine (41.9%); a polymer, glycogen (41.1%) and a carboxylic acid, pyruvic acid (36.8%). The carboxylic acid, itaconic acid (38.3%) was significantly (P<0.05) in association with samples from center. The amino acid L-arginine (36.9%) was associated with south. The phenolic compound, 2-hydroxy-benzoic acid (38.1%) was related with samples from west site. In pelagic zone two substrates were significantly (P<0.05) related: amino acid, glycil-L-glutamic acid and carboxilic acid, pyruvic acid methyl ester, both representing 37.3% of indicator value. D-galacturonic acid (36.8%), a carboxylic acid and Tween 40 (35.8%), a polymer were associated with samples from east side. These results represent the functional heterogeneity of carbon utilization varying for all sampling sites at Lake Mangueira, when investigating all seasons together.

Within summer

During summer the kinetic profile measured by the asymptote (*K* parameter) and by the *r* value did not showed significant space variation. Although the Shannon diversity index of substrate diversity showed slight higher values (data not shown) in center, north and east side of Lake, this difference was not significant. Also, the efficiency of substrate consumption (PSUE) did not show any variation. Following these results, none substrate was significantly (P<0.05) associated with any site of the Lake.

Within autumn

The sites during autumn did not show variation of *K* and *r* parameters. H' was mostly of the time very constant for all sites in the Lake. However, PCA ordination of proportional

substrate consumption efficiency (PSUE) data significantly (P<0.001) separated north from center along axis 1 (accounted 28.05% of the variation). Furthermore, along axis 2 (accounted for 18.22% of variation), north and center were significantly separated from south (Fig. 6a). In addition, two-way MANOVA (Table 5) revealed significant (P<0.05) variation between sites when verifying these relationship. North, center and south also presented different indicative substrate. Four substrates were significantly related with south: two carboxylic acids and two amino acids; four with north, two carbohydrates, one polymer and one amine; and two with center, an amino acid and a carbohydrate. The percentage of perfect indication was higher in center with L-Phenylalanine indicating 58% and in north with D-,L-α-glycerol phosphate indicating 54.2%. Some indicative substrates presented correlation with the first two axes of the PCA and the site that it was associated (Table 5).

Within winter

The kinetic profile showed spatial variation. In this way, the maximum degree of substrate utilization (*K* parameter) differed between south and center of Lake (P=0.017), having south higher average values (*K*=1.12 ± 0.03) than center (*K*=0.97 ± 0.03). The rate of AWCD change (*r* parameter), significantly (P=0.006) differed between north (*r*=-0.038±0.00) and south (*r*=-0.029±0.00). The H' index at all time of incubation presented higher values in south and north than center. At day 8 of incubation (192h) center was significantly (P<0.05) minor than both north and south. On the final of incubation (240h - 288h), this difference was not significant any more. The ordination reflected a variation of efficiency substrate consumption along the first two axis (accounted 47% of total variation) between north and center, different from that variation observed for the kinetic profile, however, only axis two (22% of variation) was significant (P<0.001) (Fig. 6b). The *two*-way MANOVA confirmed this observed relationship (P=0.011), revealing north having higher carbon consumption than center. Thus north presented three indicators substrate, belonging to two carbohydrates and

one polymer, and center presented one carbohydrate and one polymer (Table 5). Despite east side did not show any variation for the others parameters; this site presented an indicator carbon source, which was the D-Galacturonic acid, a carboxylic acid. This is the same substrate found for this site in the analysis above, when investigating all spatiality, so probably samples from winter mainly contributed for the preview result.

Within spring

Gradients in space were also observed in spring between the littoral zones of the Lake. The exponential rate of color development (*r* parameter) was significantly ($P<0.05$) higher in average in west side ($r=0.04\pm0.00$) and in pelagic zone ($r=0.04\pm0.00$) than in east side ($r=0.033\pm0.00$). The substrate diversity as well varied significantly ($P<0.05$) among pelagic zone and east side along the time of incubation, however, by the end of incubation, the difference was not significant any more, despite the H' still showed higher values on west side and pelagic zone than east side. The variation of proportional substrate utilization, accessed by PCA ordination was significantly ($P<0.001$) explained by the first two axis of PCA (accounted 54.9% of variation, 29.5 and 25.4%, respectively). For this data, the variability occurred both for extremes as for littoral (Fig. 6c and d.). Both center and north looked very similar and shared differences with south which presented upper efficiency of consumption (Fig. 6c). As summarized for kinetic profiles, the variation among pelagic zone and east side was also observed for PSUE data (Fig. 6d.) and the *two-way* MANOVA validated this distinction. Also, corroborating those variations, the indicator substrate analysis revealed two indicator carbon sources of south (an amine and a carbohydrate); a carbohydrate in center and in north; and an amino acid and a carboxylic acid in east side (Table 5).

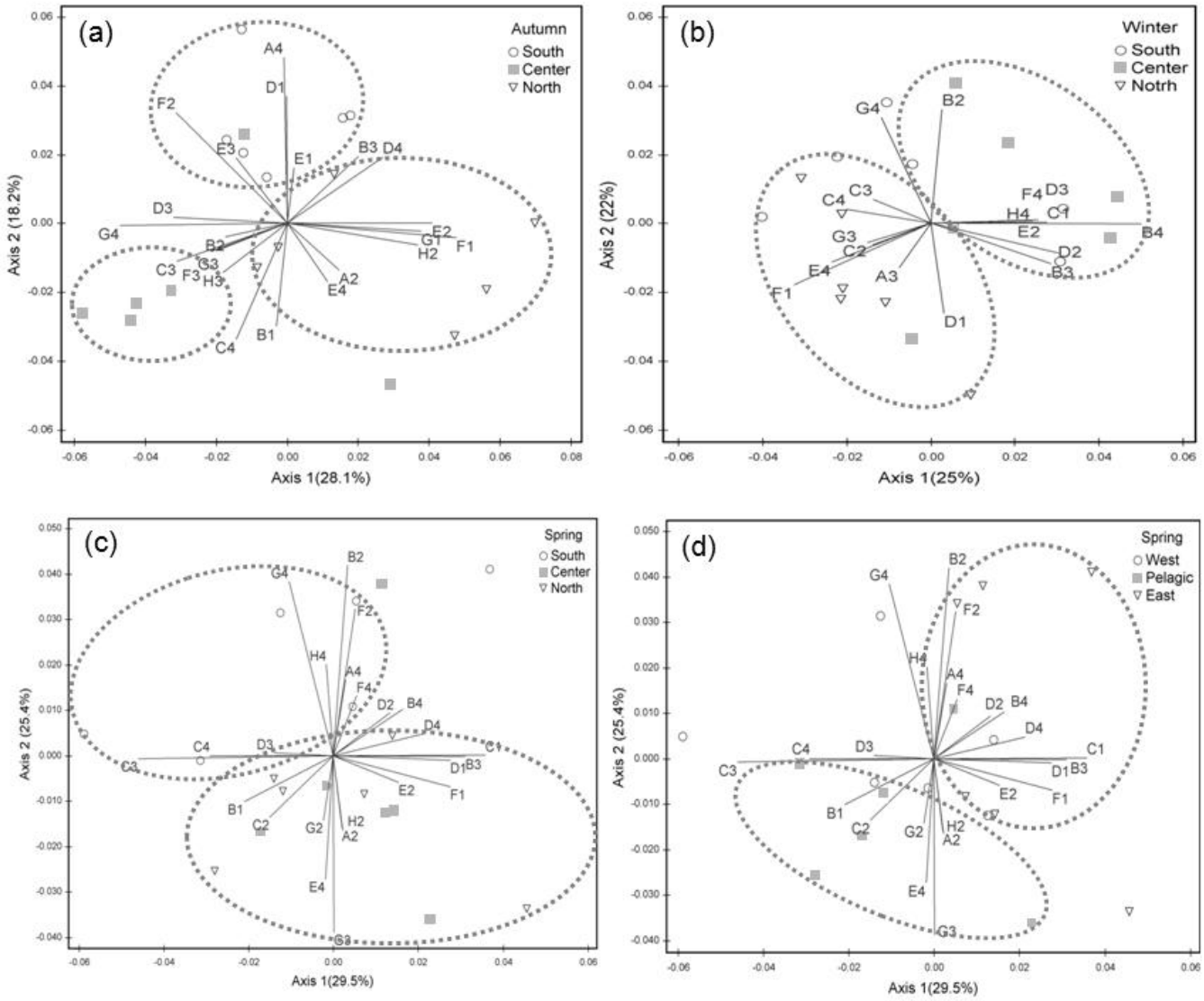

Fig. 6 PCA biplot diagram of spatial variation of PSUE data in (a) autumn, (b) winter, (c) spring, among extremes and (d) spring among littoral zones at Lake Mangueira.The carbon sources name is displayed in Table 5.

Table 5. Main results of correlation coeficients for scored of the first two principal component analysis (PCA), of indicator substrate analysis and of *two-way* MANOVA from efficiency of substrate consumption data in each season.

Carbon Sources		Autumn					Winter					Spring				
		Correlation		Indicator Substrate			Correlation		Indicator Substrate			Correlation		Indicator Substrate		
	Code	Axis 1	Axis 2	Site	% of perfect indication	*P-values*	Axis 1	Axis 2	Site	% of perfect indication	*P-values*	Axis 1	Axis 2	Site	% of perfect indication	*P-values*
Amines/amides																
Phenylethylamine	G4	-0.821						0.714					0.799	south	46	0.011*
Putrescine	H4			north	40.2	0.032*	0.611						0.581			
Amino acids																
L-Arginine	A4		0.828	south	40.9	0.049*							0.528			
L-Asparagine	B4						0.912					0.523				
L-Phenylalanine	C4		-0.693	center	58.3	0.005**	-0.576					-0.697				
L-Serine	D4	0.619		south	38.2	0.046*						0.598				
Glycyl-L-glutamic acid	F4						0.65							east	39.1	0.041*
L-Threonine	E4		-0.491				-0.628						-0.674			
Carbohydrates																
α-D-lactose	H1															
β-Methyl-D-glucoside	A2												-0.515			
D-Cellobiose	G1	0.733														
D-Mannitol	D2						0.712		center	35.7	0.05*					
I-Erythritol	C2						-0.502		north	44.8	0.042*	-0.499				
Glucose-1-phosphate	G2												-0.486	center	41.9	0.02*
D-Galactonic acid -Υ-lactone	A3															
N-Acetyl-D-glucosamine	E2	0.762		north	41.8	0.043*	0.585					0.508				
D,L-α-glycerol phosphate	H2	0.724		north	54.2	0.002**			north	44.7	0.031*		-0.523	north	44.6	0.025*
D-Xylose	B2	-0.501						0.741					0.837	south	42.7	0.043*
Carboxylic & acetic acids																
α-ketobutyric acid	G3	-0.554					-0.48						-0.804			
D-Galacturonic acid	B3	0.534	0.525	south	40.7	0.037*	0.689		east	36.9	0.032*	0.721				
D-Glucosaminic acid	F2		0.676	south	44.7	0.006**							0.734	east	38.4	0.038*

Continuation of Table 5

Carbon Sources		Autumn					Winter					Spring				
		Correlation		Indicator Substrate			Correlation		Indicator Substrate			Correlation		Indicator Substrate		
	Code	Axis 1	Axis 2	Site	% of perfect indication	*P-values*	Axis 1	Axis 2	Site	% of perfect indication	*P-values*	Axis 1	Axis 2	Site	% of perfect indication	*P-values*
ϒ-Hydroxybutyric acid	E3		0.519													
D-Malic acid	H3	-0.508														
Pyruvic acid methyl ester	B1		-0.654									-0.591				
Itaconic acid	F3	-0.534		center	46.6	0.009**										
Polymers																
α-cyclodextrin	E1		0.48													
Glycogen	F1	0.825		north	41.4	0.013*	-0.735		north	46.5	0.018*	0.68				
Tween 40	C1						0.676		center	36.9	0.016*	0.774				
Tween 80	D1		0.724					-0.658				0.678				
Phenolic Compounds																
2-Hydroxy benzoic acid	C3	-0.666					-0.48					-0.875				
4-Hydroxy benzoic acid	D3	-0.678					0.633					-0.483				
two-way MANOVA		P-values:														
Extreme				0.001 **					0.04 *					0.032 *		
South X North				0.015 *					ns					0.044 *		
South X Center				0.013 *					ns					0.018 *		
Center X North				0.028 *					0.011 *					Ns		
Edge				0.24 ns					0.53 ns					0.019 *		
West x Pelagic				ns					ns					ns		
West X East				ns					ns					ns		
Pelagic X East				ns					ns					0.018 *		
ExtxEdge				ns					ns					ns		

CD, code of substrates. Correlation value>0.48 are significant (P<0.05). Indicator substrate analysis and *Two-way* MANOVA *P≤0.05; **P≤0.01; ns, not significant (n=18).

3.3.Limnological Data Driving Carbon Substrate Utilization

The relationship between environmental factors and consumption parameters were observed for seasonal and spatial changes. The kinetic profile showed correlations for overall seasons and within each season (Table 6). The RDA results were only significant for overall season, within autumn, within winter and within spring. The relationships between limnological data and functional attributes during summer were not significance in this analysis. The main results for the first two axes of RDA were summarized in table 7.

Seasonality of functional heterogeneity

The seasonality of kinetic parameters using the full data set (n=72) showed well correlated with most of the environmental data (Table 6). *K* value showed significant ($P<0.05$) positive relationship with SRP, and negative with TN, TOC, DOC and Secchi. The rate of AWCD change (*r* value) was positively correlated with TSS, TP, Chl*a*, pH, and temperature and negatively correlated with secchi. Only turbidity did not show correlation with *K* and *r*. The Pearson's product-moment correlation between the PSUE data and the environmental variables resulted 25 of the 31 substrates significantly ($P<0.05$) correlated with at least one (except ammonium which did not correlated with any one) variable. These results were then analyzed in the RDA.

According to Fig. 7, the RDA presented a slightly but significant temporal separation (accounted 12.5% of total variation of first two axis; $P=0.002$) between carbon consumption and limnological data. Along axis 1, TN ($r=0.61$), TOC ($r=0.41$), DOC ($r=0.39$), pH ($r=0.40$), and nitrate ($r=-0.30$) were the mainly variables that contributed to the separation of close season (summer and autumn) from the others (winter and spring). The separation along axis 2 of the majority of samples from summer and autumn were driven by water temperature ($r=0.36$), TP ($r=0.25$) and SRP ($r=0.38$). Separation of samples from winter and spring were

driven mostly by, Secchi (r=-0.50), nitrate (r=-0.32) and also SRP (r=0.38). The majority of indicator substrate were related to the season that represents. The two amino acids (L-Arginine (A4), r=-0.13 and L-Serine (D4), r=0.51) and the amine (Putrecine (H4), r=0.38), were correlated with the first and second axis with samples from summer. ϒ-Hydroxybutyric acid (E3), a carboxylic acid which represented a good percentage of indication during summer, and was not consumed during winter and spring showed positively correlated to TSS, Chl*a*, TN, TOC and pH. However, in the Fig.7 it was related with summer and autumn, along axis 1, not exclusively with summer.

Table 6. Pearson product-moment correlation between metabolic kinetic profile and limnological data on the overall seasons and within each season at Lake Mangueira.

	Overall Seasons		Summer		Autumn		Winter		Spring	
	K	R	K	r	K	r	K	R	K	r
Turbidity	0.16	0.21	0.23	0.09	0.55*	0.36	0.08	0.58*	0.42	0.85***
TSS	0.11	0.28*	-0.02	-0.08	0.54*	0.47*	0.00	0.35	0.47	0.83***
TP	-0.06	0.42***	0.00	0.17	0.42	0.27	-0.16	0.24	0.13	0.69**
SRP	0.45***	0.21	0.24	0.25	0.36	0.17	0.22	0.35	0.50*	0.74***
TN	-0.30**	-0.01	-0.10	-0.28	-0.38	-0.30	0.43	0.19	-0.04	0.24
Chl*a*	-0.04	0.28*	0.23	-0.05	0.44	0.27	-0.07	0.50*	0.39	0.76***
TOC	-0.33**	-0.22	0.20	-0.08	0.08	0.35	-0.14	-0.22	-0.40	-0.40
DOC	-0.28*	-0.18	0.07	-0.08	0.06	0.30	0.16	-0.13	-0.29	-0.10
Secchi	-0.26*	-0.27*	-0.16	0.06	-0.61**	-0.50*	-0.10	-0.69**	0.50*	-0.76***
pH	-0.20	0.35**	0.22	0.76***	0.27	0.04	0.06	-0.75***	0.29	0.08
Temp	0.11	0.35**	na	na	na	na	na	na	na	na

TSS, total suspended solids; TP, total phosphorus; SRP, soluble reactive phosphorus; TN, total Nitrogen; Chl *a*, chlorophyll *a;* TOC, total organic carbon; DOC, dissolved organic carbon; Temp, water temperature. K, asymptote of AWCD r= rate of AWCD change. Pearson's correlation *P≤0.05; **P≤0.01; ***P≤0.001; na= not analyzed.

The three indicator substrates of autumn, Tween 80 (D1) (r=0.36), Glycogen (F1) (r=0.36) and D-cellobiose (G1) (r=0.45) were related with samples of this season along axis 1. Glycogen presented higher percentage of perfect indication (30.4%), however, in this analysis, this substrate appear to not be the most correlated with samples from autumn. D-Mannitol (D2) r=-0.68, a carbohydrate, related to axis 2, showed significant potential of indication during winter and it was positively correlation with water transparence and

negatively correlation with temperature, which probably explain the higher consumption of this carbohydrate during this season. I-Erythritol (C2), r=-0.56, another substrates in association with winter (40% of perfect indication), presented well correlated with this season in this analysis. In spring, the α-Ketobutyric acid (G3) showed 35% of perfect indication and was slightly correlated with samples from this season in RDA.

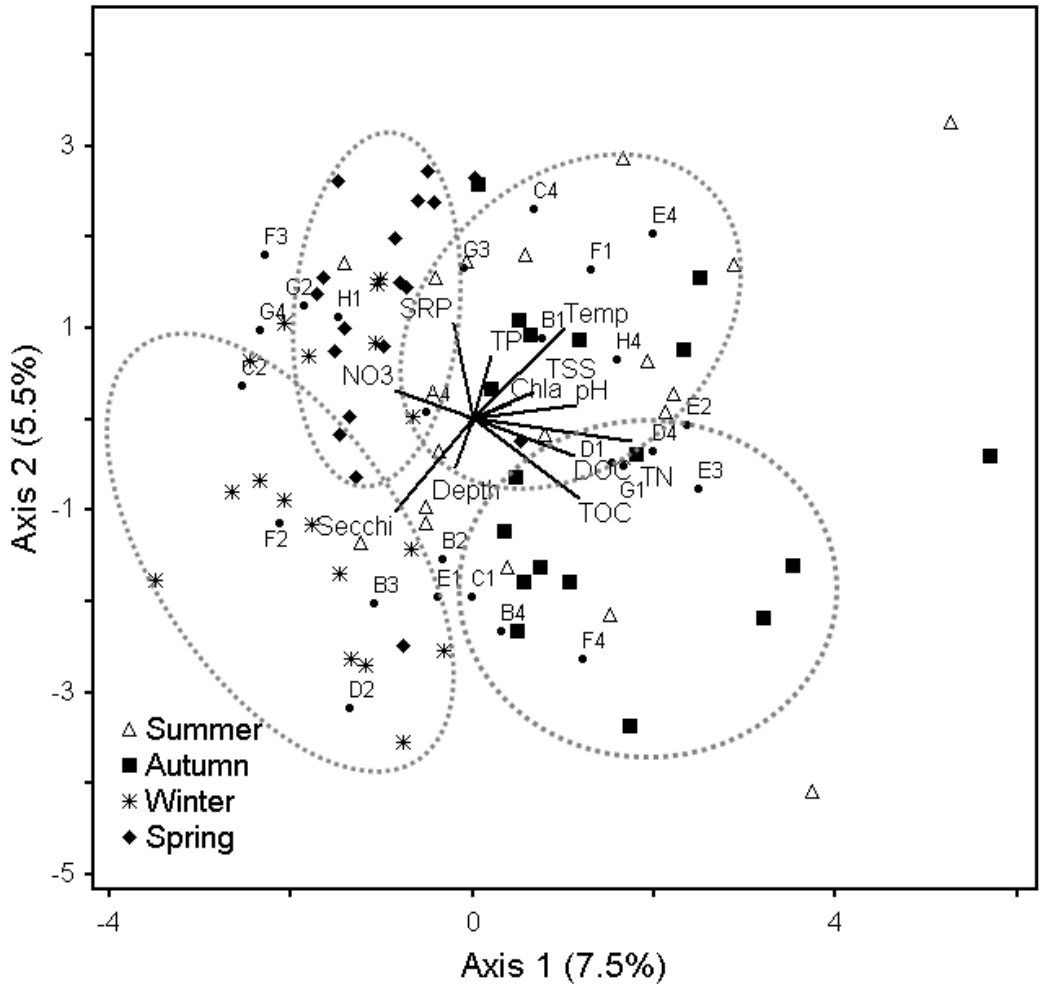

Fig. 7 RDA triplot diagram between PSUE and limnological data of Lake Mangueria during overall season. The name of the carbon sources are displayed in Table 5.

Spatial heterogeneity

The kinetic approach was correlated with some environmental variable, when investigating correlations within each season (Table 6). Summer presented only correlation between the rate of AWCD change (*r*-value) and pH. The *r*-value was negatively correlated with secchi, during autumn, winter and spring. In autumn, *K* and *r* presented positive correlation with TSS. In winter and spring *r*-value was positive correlated to Chl*a* and turbidity. Spring showed the majority of positive correlations of *r*-value for production component and nutrients dynamics represented by turbidity, TSS, SRP, TN and Chl*a*.

The relationship between the proportional substrate utilization efficiency and the limnological characteristics assessed by the RDA confirmed some spatial variation across the extremes of the lake, during winter, autumn and spring. For these seasons, this analysis was significant ($P \leq 0.05$). The RDA did not show any clear ordination between samples comparing littoral zones (data not shown) for any season. Summer, as expected by preview results, did not show significance for the RDA analysis, which means that observed results of substrate utilization and environmental data still not display any spatial trend during summer.

Autumn showed spatial separation across the first two axis of the RDA ordination (accounted 48.2% of total variation; P=0.006). As presented in Fig 8a, along axis 1 (28.8%), center and south were separated from north. Along axis 2 (19.4%) center and south were separated from each other. The separation of central area from the others was controlled by turbidity (r=0.37) and TSS (0.51) along axis 1, SRP (r=0.47) and pH (r=0.59) along axis 2, which influenced the consumption of L-Phenylalanine (C4), an indicator substrate of the center. In preview analysis, this substrate was found to be significantly correlated with turbidity ((r=0.52) and secchi (r=-0.49). In north side, ammonium (r=-0.77), nitrate (r=-0.65) and secchi (r=-0.62) shaped the consumption of D-L-α-glycerol-phosphate (H2, r=-0.85) and

Glycogen (F1, r=-0.82), both indicators of this site. Also the consumption of (β-metil D-glucoside (A2) (r=-0.75), was influenced by these parameters in north. South had no gradient of environmental data relating to the functional attributes. However, the two amino acid, L-arginine (A4) and L-serine (D4) that shared characteristic of potential indicators of south were correlated to this site in this analysis.

The RDA ordination for winter accounted 39.1% of total variation for first two axes (Fig. 8b). The ordination significantly (P=0.048) revealed spatial separation within the Lake. Ammonium (r=0.38), Chl*a* (r=0.62), Turbidity(r=0.67), SRP (r=-0.45), TN (r=0.36), TSS (r=0.51) and nitrate (r=0.45) displayed a gradient that increased toward north area. The indicators substrates of north, two carbohydrates (I-erythritol - C2 and D-L-α-glycerol phosphate - H2), and a polymer (glycogen - F1) were well correlated with samples from this site. Substrates F1 and C2 were the most correlated with north, also presented higher percentage of perfect indication, 46.5 and 44.8%, respectively. Other substrates that did not presented potential of indication also contributed to the gradient toward north area: a carbohydrate (D-galacturonic acid - A3), two amino acids (Glycyl-L-glutamic acid - D4 and L-Threonine - E4) and one polymer (Tween 80 - D1). Center and south were correlated with pH (r=0.68), DOC (r=0.18), secchi (r=0.67) and depth (r=0.71) along axis 1. Tween 40 (C1) a substrate associated to center, was positively correlated with depth (r=0.49), and negatively with TN (r=-0.65) and NO_3 (r=-0.65). Other substrates, as D-Xylose (B2), 4-Hydroxy benzoic acid (D3) and Putrecine (H4) also correlated to the central area being influenced by those parameters.

The RDA analysis of spring (Fig.8c), significantly (P=0.05) explained 46.3% for the first two axes, 26.8 and 19.5%, respectively. Mainly sample of north were clearly influenced by TP (r=-0.65), Chl*a* (r=-0.62) and TSS (-0.54) along axis 1. The utilization of D,L-*α*-glycerol phosphate (H2) (r=-0.72), north indicator; was slightly related with samples from

north, along axis 1. The majority of samples from central area were related to ammonium (r=0.60), depth (r=0.20), DOC (r=0.34) and TOC (r=37). Here the carbohydrate, Glucose 1-phosphate (G2), seems to not contribute so well for the distribution of samples in the ordination; however this substrate was also an indicative substrate of central area during spring. In south, pH (r=-0.39) displayed a gradient that increased toward this site, and probably drove the utilization of Phenylethylamine (G4) in south.

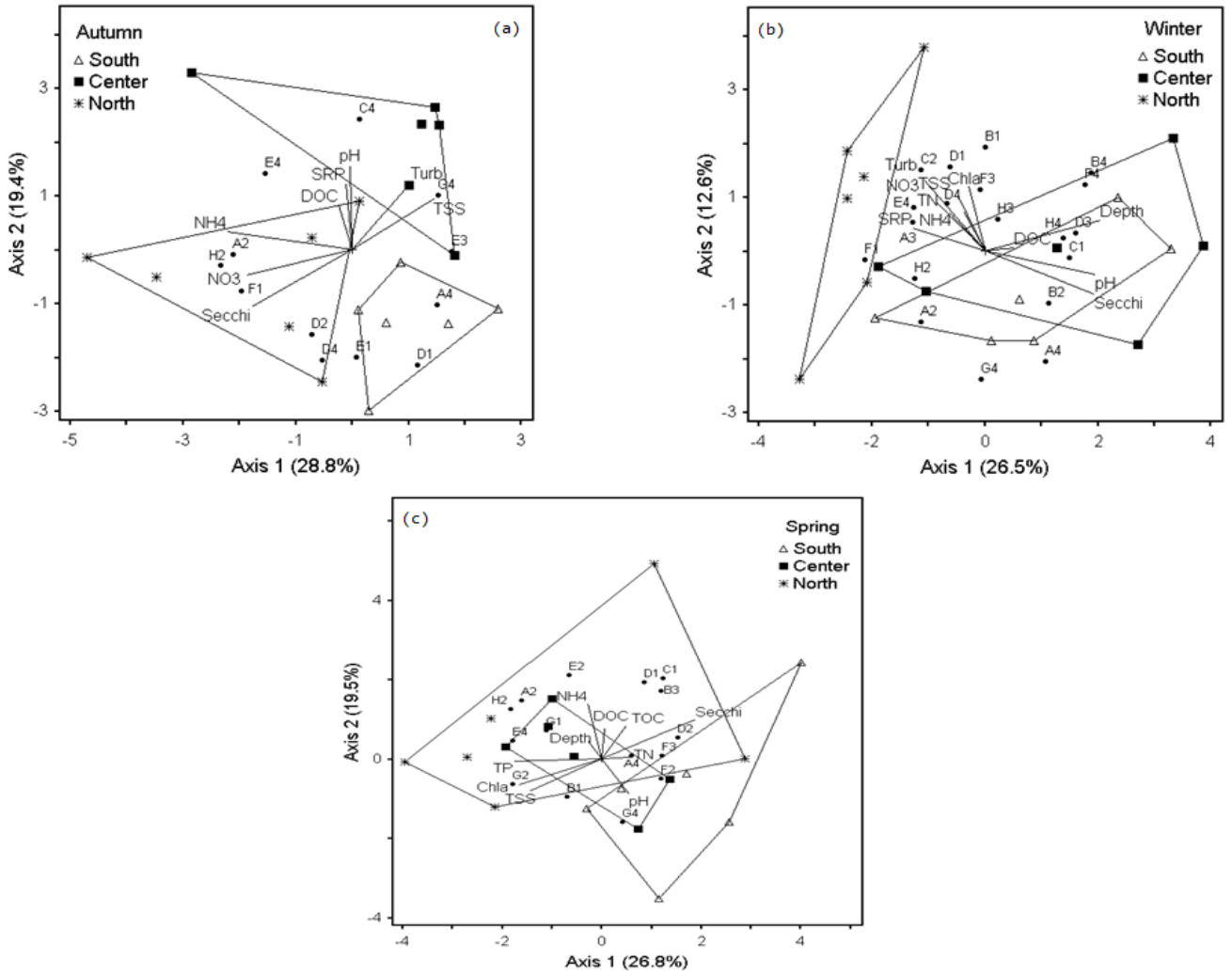

Fig. 8 RDA triplot diagram with functional attributes and limnological data during (a) autumn (b) winter and (c) spring at Lake Mangueira. The name of the carbon sources are displayed in Table 5.

Table 7. Main results of the RDA ordination for overall season and within autumn, winter and spring at Lake Mangueira.

	Overall Season		Autumn		Winter		Spring	
	Axis 1	Axis 2	Axis 1	Axis 2	Axis 1	Axis 2	Axis 1	Axis 2
Eigenvalue	1.831	1.306	3.455	2.325	5.035	2.398	4.022	2.921
Percentage of variance explained	7.3	5.2	28.8	19.4	26.5	12.6	26.8	19.5
Cumulative % explained	7.3	12.5	28.8	48.2	26.5	39.1	26.8	46.3
Inertia (total variance)	25.00		12.00		19.00		15.00	
Significance test	0.002**		0.006**		0.048*		0.05*	
Intra-set correlations biplot								
Turb			0.37	0.47	-0.38	0.67		
TSS	0.24	0.10	0.51	0.36	-0.17	0.51	-0.54	-0.36
TP	0.07	0.25					-0.65	-0.02
SRP	-0.07	0.38	-0.04	0.47	-0.45	0.22		
TN	0.61	-0.09			-0.23	0.36	0.21	0.02
NH_4			-0.77	0.123	-0.13	0.38	-0.10	0.60
NO_3	-0.30	0.11	-0.65	-0.18	-0.30	0.45		
Chla	0.15	0.06			-0.11	0.62	-0.62	-0.28
TOC	0.41	-0.32					0.18	0.37
DOC	0.39	0.27	-0.09	0.31	0.18	0.03	0.02	0.34
Depth	-0.07	-0.20			0.71	0.29	-0.11	0.20
Secchi	-0.30	-0.37	-0.62	-0.40	0.67	-0.41	0.70	0.44
pH	0.40	0.05	-0.01	0.59	0.68	-0.22	0.20	-0.39
Temp	0.35	0.36						

Definition of limnological variables see footnote of table 6.

4. Discussion

4.1. General Trends of the Limnological Scenario

It was evidenced that Lake Mangueira presented a seasonal and spatial heterogeneity related to its environmental characteristics and microbial functional attributes. As found by Rodrigues (2009), in Lake Mangueira, the seasonality of limnological data was driven mainly by nutrients (TP, TN and Nitrate) dynamics, by Chl*a*, and TSS contributed for summer separation compared to other seasons. Despite all season showed high variation between each other, we could observe that summer and autumn were slightly more closely between each other as in the same way, winter and spring shared similarities.

Spatial heterogeneity displayed by limnological characteristics was observed mainly for autumn, winter and spring; in which south and north constantly differentiated from each other. Also, center of Lake Mangueira shared some differences in relation to south and north. Trends across north and south were also evaluated by Rodrigues (2009) in Lake Mangueira, where nutrients, turbidity and production component were associated mostly to north, maybe due to inputs of nutrients (phosphorus and nitrogen) and organic compounds from Taim wetland. Whereas in the south the higher water transparency and minor chlorophyll *a* concentration were consequence of presence of submerged macrophytes (Finkler-Ferreira, 2009) inhibiting phytoplankton production (Rodrigues, 2009). Another hypothesis for this spatial trend is the same found by Cardoso and Motta Marques, (2009) in a similar subtropical, coastal and shallow lake. They reported that northeast winds displaced water from north to south, playing an important role in driving physical variables. In Lake Mangueira wind velocity reaches 90km/h, displacing water, predominantly, from northeast to southwest (Fragoso Jr. et al 2008), probably explaining the spatial variation found across these sites in our study

The lack of significant differences among south and north during summer is probably due to low depth, which influenced resuspension events, turning the Lake a homogenously mixture in both extremes.

Winter and spring showed major differences among littoral zones. In west zone was observed higher average concentration of nutrients in winter and total solid suspense in spring. According to Motta Marques *et al*. (1997), in this zone, Lake Mangueira is surrounded by emergent and submerged macrophytes and rice field that release organic compound and nutrients, respectively into the Lake, and thus explaining these differences.

The observed trends across Lake Mangueira demonstrate a possible occurrence of two stable alternative states as observed by Finkler-Ferreira *et al.* (2008) also in this Lake. According to Jeppensen, *et al*. (1997), these alternative states, in shallow lakes, are associated to water transparency or turbidity, and consequently to the trophic cascade interactions in the system. Thus the functional attributes of microbial community could be shaped by these alternative states. As observed below the utilization of carbon sources by microbial community varied according to water condition, as transparency and turbidity.

4.2. Seasonality and spatiality of overall functional potential

Despite this method present some limitations, as inoculums density (Garland, 1997; Preston-Mafham et al 2002), bias in fast-growing, and the substrate of aquatic system be more complex than those in Ecoplates (Smalla et al 1998), what may not represent all community members, although the presence of rare members could be detected (Haack et al 1995). To avoid such limitation, we analyzed the data according Guckert *et al.*, (1996); Lindstrom *et al.*, (1998); Pietikäinen *et al.*, (2000).

Using sole carbon source approach we were able to generate different kind of data that revealed most of the functional potential of heterotrophic aquatic microbial community on both overall (AWCD data) and more restrict (each 31 substrate) views. Physiological profile assessed by Biolog Ecoplate assay has shown that the functional diversity of aquatic bacterial assemblages differ on both temporal and spatial scales in Lake Mangueira. Spatial and temporal variation of functional attributes was also observed in some studies around the world in aquatic systems (Grover and Chrzanowski, 2000; Sala *et al*., 2006, 2008; Christian and Lind, 2007; Tiquia, 2010).

The overall metabolism varied across season. In spring, the degree of consumption of AWCD (*K*) was high and so was the velocity of carbon metabolism (*r*), both on spring and summer. Maybe, the high value of *K*, in fact, represents that, in spring, the microbial functional potential, at Lake Mangueira, reached the highest carrying capacity (Lindstrom *et al*, 1998). The trend observed for *r* is probably explained by differences of in situ water temperature - which could be an important factor for seasonal variation in biological activity. This hypothesis takes account that bacterioplankton production is also affected by temperature (Vrede, 2005). In this way warm temperatures allow optimum growth condition for several bacteria (Madigan et al., 2004). Also, according to Grover and Chrzanowski (2000), opportunistic bacterioplankton increase its abundance when the relative supply of substrate is seasonally elevated. In addition, differences in substrate availability may enhance various opportunities of catabolism pathway (Madigan et al., 2004).

The spatiality was observed during winter when north presented high *r* than south due to positive correlation with Chl*a* and turbidity found in north and negative correlation with secchi and pH in south. In contrast, spring presented spatial differences for *r* across the littoral zones, driven by nutrients, Chl*a* and turbidity which increased *r*

in west side and in pelagic zone compared to east side where water transparency inhibited the velocity of carbon metabolism. This trend reflects how the whole functional structure of microbial community, in Lake Mangueira is influenced by what surround it. In North, Taim wetland release many nutrients and organic compounds into the Lake (Motta-Marques *et al*., 1997; Finkler-Ferreira *et al*., 2008; Rodrigues, 2009); in south, submerged macrophytes inhibit phytoplankton and bacterial production (Rodrigues, 2009); in west are presented emergent macrophytes and rice fields which also release nutrients; finally, in east is the ocean side with fixed dunes which influence the transparency of water. The spatiality of kinetic parameters was not evident during summer and autumn probably due to Lake Mangueira characteristics was mostly homogeneous in summer due ressuspention events and this also reflected in autumn results.

Both *K* and *r* appear to be affected by synergistic and antagonistic effects due to the mix and the taxonomic richness of microplate-culturable organism and their relative abundance, but not on inoculums density (Lindstrom *et al.*, 1998). Therefore parameter *K* represents the asymptote of the modeled absorbance curve and the *r* parameter is an estimate of the exponential rate of color development, which provides information on how rapidly a carbon source can be metabolized by a community once the density has reached the level at which color production begins. Thus, corroborating Lindstrom *et al*. (1998) and Prestom-Mafham *et al*. (2002), *r* may be the most useful parameter for comparing the relative functional responses of different communities.

Differences in Shannon diversity index somehow reflected the results obtained for *K* parameter of kinetic profiles. In spring occurred higher average value of diversity during incubation time. Only during the exponential growth, H' in winter shared similarities with spring. Also, both seasons were the only which presented spatial

variation for this data. Probably the higher diversity during spring reflects the increase in the metabolic activity of microbial population or could be consequence of existence of rare species, which can have a large effect on substrate utilization patterns (Zak *et al.*, 1994) exerting a large activity in certain wells (Weber *et al.*, 2008). The lack of spatiality in substrate diversity during summer and autumn, may reflect that the microbial ecology at this time were more robust dealing with Lake characteristic more readily, as found by Weber *et al*.(2008).

4.2.1. Restrictive view of Physiological profile within time and space

Seasonality

The substrate indicator analysis following Dufrêne and Legendre (1997) was efficient in reveal which substrate was potentially utilized among and within season, considering that this analysis was based on within-substrate relative abundance and occurrence comparisons and that its values is not affected by the relative abundance of other substrates. Although summer did not show any variation for overall metabolic diversity (AWCD and Shannon diversity), samples from this season were significantly associated with more substrates rather than autumn, winter and spring. According to Salomo *et al.* (2009), the pure butyric acid is a short-chain fatty acid generally easy to be degrade, but with hydroxyl group, turns a *y*-Hydroxybutiric acid a biochemically inert compound, which is hard to be degrade, suggesting that during summer some microbial specialists settle in these substrates. In addition, this substrate was not utilized by any sample from winter and spring, what indicates that along the season, Lake Mangueira presented distinct community based on functional attributes (Colwell and Lehmam, 1996). On the other hand, none phenolic compound appear to be potentially relevant utilized for any season, what suggests that communities with same functional attributes also occur for overall season. Amino acids and amine are groups with N-

containing, and its consumption were associated with summer, suggesting that bacteria could be nitrogen deprived (Christian and Lind, 2007) during this period. Amino acids play an important role for growth of heterotrophic bacteria, since they comprise 60% of their biomass (Simon and Azam, 1989). Polymers and carbohydrates represent energy sources for bacteria and were well documented during autumn and winter. In this way, Grover and Chzarnowski (2007) suggest that high utilization of carbohydrates is driven by variance in algal abundance, and transfer process as exudation, lyses and grazing. Spring and summer, presented a utilization of different derivate of butyric acid, the ketobutyric acid, which may also suggest some specialization of these attributes of microbial group due to the hardness in degradable this substrate (Salomo *et at*., 2009). According to Bertilsson and Tranvik, (2000) carboxilic acid are often naturally a product of bacterial fatty acid catabolism, photochemical degradable.

All substrate in association with overall season presented correlation with some environmental data and thus were well documented that the bacterial functional potential were manly drove by this characteristics among the seasons as demonstrated in RDA (Fig. 7). Although this analysis showed a low percentage of explanation, the distribution of samples into the diagram was significant. Indeed the closely samples (summer plus autumn and winter plus spring) were also closely in the diagram across RDA1.

Spatiality

Autumn, winter and spring displayed differences related to the utilization preference of 31 carbon sources between sampling sites. In contrast, this trend was not evident during summer, neither when analyzing indicative substrates occurring among sites, nor when looking for limnological drivers within this season in the RDA analysis.

We suppose that heterogeneity in substrate utilization between sites in autumn, winter and spring were related to dissimilarities in limnological variables across Lake Mangueira.

In autumn, differences between center and north could be explained by concentration of Chl*a* in center, suggesting that physiological profiles assessed in this site rely mainly on phytoplankton interactions (Sala *et al.*, 2008). Wetland metabolism and epiphytic and detrital microflora associated, control much the inputs of nutrient within the water body (Wetzel 1992) such as the case of Taim Wetland, contributing eventually to shifts of functional attribute, where putrescine, N-acetyl-d-glucosamine, D,L-*a*-glycerol phosphate and glycogen (i.e. amine, carbohydrate and polymers, respectively) were in association with this site. The relationship with polymer was also found by Werh *et al*. (1999), in a Mesotrophic Lake, where sugar and carboxylic acids were metabolized to a greater extent when supplemented by macrophyte detritus. In addition, Cunningham and Wetzel (1989) indicates that mineralization of proteins by wetland sediment microflora conformed to kinetic of polymers degradation.

The substrates which contributed to differentiation of south from center and north, maybe, were influenced by a negative control of Chl*a*, turbidity and nutrient dynamic, since none of those parameters were positively related to the utilization of those substrates in south. In addition, SRP was significantly negatively correlated to α-cyclodextrin (polymer), which was related to south in autumn; and as well known phosphorus is important in limiting bacterioplankton production (Vrede, 2005).

Differences during winter, in relation to north and center, also revealed that nutrient dynamic, Chl*a* and turbidity concentration influenced, in north, the functional attributes, whereas, water transparency, depth, pH and DOC influenced the carbon

consumption in center. Loading of DOC from extracellular releases from phytoplankton is commonly assumed to be a major source for bacteria in pelagic zones and in these zones most decomposition is made by free-living bacteria that utilize organic compounds (Wetzel, 1992), suggesting that microbial community could by compositionally and metabolic different in center and north, thus influencing the physiological profile.

In spring, we found Phenylethylamine, an aromatic amine which present a highly basic pH, in strong association with south, which also presented basic pH characteristics. The utilization of Glucose 1-phophate was well documented for center of the Lake, in spring, and it was related to concentrations of total phosphorus. Glucose-1-phosphate can be easily converted in glucose-6-phophate, a central key for glycolysis process, in which, each molecule of glucose fermented, two molecule of ATP are generated (Madigan et al., 2004). Thus, the use of this substrate was related to source of energy for the community.

D,L-*a*-glycerol phosphate was found in association with north during autumn, winter and spring, suggesting that despite shifts on utilization preference of carbon source, we were able to identify persistence of functional attributes of bacterial assemblages across time and space and independently of water characteristics.

This study demonstrated that particular substrates were potentially relevant between and within season. However, these trends may be a result from the interaction between environmental data and microbial functional attributes from few culturable groups (generalists), in which a population of opportunistic bacteria with different substrate capability vary in abundance over the growing season (Grove and Chrzanowski, 2000) or maybe a largely distributed among taxonomic groups (many

specialists) (Colwell and Lehman, 1996). Also, as well documented by Sala *et al*. (2006) in Mediterranean Sea, heterotrophic microbial communities in oligotrophic system adapted to constants changing inputs of nutrients and organic compounds and thus, in order to exploit these changes and the scarce carbon source available for growth, they may be expressing a high plasticity in metabolic pathways.

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Appendix 1. Space variation within each season of each limnological data at Lake Mangueira.

Seasons \ Sites		Turbidity (NTU)	TSS ($mg.l^{-1}$)	TP ($mg.l^{-1}$)	SRP ($mg.l^{-1}$)	TN ($mg.l^{-1}$)	Chl*a* ($ug.l^{-1}$)	TOC ($mg.l^{-1}$)	DOC ($mg.l^{-1}$)	Depth (m)	Secchi (m)	pH
Summer	Extreme	0.119 ns	0.496ns	0.252 ns	0.045 *	0.94 ns	0.044 *	0.194 ns	0.41 ns	0.286 ns	0.217 ns	0.005 **
	SxC	Ns	ns	ns	0.021 *	ns	0.025 *	ns	ns	ns	ns	ns
	SxN	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.022 *
	CxN	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.032 *
	Littoral	0.864ns	0.335 ns	0.884 ns	0.801 ns	0.221 ns	0.211 ns	0.609 ns	0.751 ns	0.006 **	0.487 ns	0.557 ns
	WxP	Ns	ns	ns	ns	ns	ns	ns	ns	0.007 **	ns	ns
	WxE	Ns	ns	ns	ns	ns	ns	ns	ns	0.024 *	ns	ns
	PxE	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Ext x Lit	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Autumn	Extreme	0.25ns	0.308 ns	0.081ns	0.274 ns	0.017 *	0.477 ns	0.329 ns	0.308 ns	0.226 ns	0.132 ns	0.003 **
	SxC	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.008 **
	SxN	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CxN	ns	ns	ns	ns	0.013 *	ns	ns	ns	ns	ns	0.032 *
	Littoral	0.327ns	0.21ns	0.09ns	0.331 ns	0.567 ns	0.103 ns	0.969 ns	0.759 ns	0.009 **	0.339 ns	0.566 ns
	WxP	ns	ns	ns	ns	ns	ns	ns	ns	0.011 *	ns	ns
	WxE	ns	ns	ns	ns	ns	ns	ns	ns	0.022 *	ns	ns
	PxE	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Ext x Lit	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Winter	Extreme	0.008 **	0.207 ns	0.243 ns	0.026 *	0.045 *	0.022 *	0.61 ns	0.266 ns	0.386 ns	0.002 **	0.001 **
	SxC	ns	ns	ns	ns	0.009 **	ns	ns	ns	ns	ns	0.051 /ns
	SxN	0.013 *	ns	ns	ns	ns	0.04 *	ns	ns	ns	0.013 *	0.007 **
	CxN	ns	ns	ns	0.034 *	ns	ns	ns	ns	ns	0.009 *	0.014 *
	Littoral	0.14 ns	0.263 ns	0.545ns	0.544 ns	0.01 *	0.069 ns	0.506 ns	0.639 ns	0.009 **	0.365 ns	0.755 ns
	WxP	ns	ns	ns	ns	ns	ns	ns	ns	0.021 *	ns	ns
	WxE	ns	ns	ns	ns	0.025 *	ns	ns	ns	ns	ns	ns
	PxE	ns	ns	ns	ns	0.01 *	ns	ns	ns	ns	ns	ns
	Ext x Lit	ns	ns	ns	ns	ns	0.046 *	ns	ns	ns	ns	ns
Spring	Extreme	0.013 *	0.034 *	0.002 **	0.403 ns	0.807 ns	0.017 *	0.242 ns	0.101 ns	0.309 ns	0.035 *	0.112 ns
	SxC	0.018*	ns	0.007 **	ns	ns	ns	ns	ns	ns	0.015 *	ns
	SxN	0.031*	ns	0.022 *	ns	ns	0.031 *	ns	ns	ns	0.04 *	ns
	CxN	ns	ns	0.047 *	ns	ns	ns	ns	ns	ns	ns	ns
	Littoral	0.001 **	0.004 **	0.131 ns	0.222 ns	0.716 ns	0.115 ns	0.649 ns	0.719 ns	0.011 *	0.034 *	0.448 ns
	WxP	0.031 *	ns	ns	ns	ns	ns	ns	ns	0.012 *	ns	ns
	WxE	0.013 *	0.012 *	ns	ns	ns	ns	ns	ns	ns	0.051/ns	ns
	PxE	ns	0.013 *	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Ext x Lit	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Definition of limnological variables see footnote of table 6.; S, south; C, center; N, north; W, west side; P, pelagic zone; E, east side; Ext, extreme; Lit, littoral. ANOVA *two-way* (n=18, within each season): *P≤0.05; **P≤0.01; ns, not significant.

5 CAPÍTULO 2

5.1 APRESENTAÇÃO

O capítulo a seguir apresenta as informações dos atributos funcionais da comunidade microbiana aquática, na Lagoa Mangueira. Investiga os padrões funcionais ao longo do perfil de profundidade, entre quatro estações. De forma complementar verifica as relações com as condições ambientais da Lagoa.

Weak functional stratification of heterotrophic bacteria between seasons in a subtropical shallow lake

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Abstract

Aquatic microbial communities are important components in aquatic metabolism. Seeking to understand relationships involving environmental changes and costs that these changes could promote in assemblages of microorganisms, this study was conducted in Lake Mangueira, a coastal and large subtropical shallow lake located in southern Brazil, during summer, autumn, winter and spring. The objective was to evaluate, the heterotrophic microbial metabolic diversity through consumption patterns of known carbon sources using Biolog Ecoplates^{TM}. Seasonal changes were found in water characteristics and among heterotrophic bacteria assemblage function. Summer samples appeared to be more distinct comparing to others, also seven carbon sources, mainly carbohydrates, were significantly associated to summer followed by winter that presented six substrates associated belonging to several guilds. Redundancy analysis confirmed that some of the substrates potentially indicators of summer were determined by one or more limnological variables. In winter, autumn and spring was also found relationship between environmental variables and susbstrate utilization. These season presented more proximity between its samples. Finally, it was possible to suppose that the temporal pattern of preference and use of carbon sources, were influenced by environment characteristics.

Keywords: *freshwater, seasonality, functional diversity, Ecoplate.*

1. Introduction

In freshwater lakes, heterotrophic bacteria are the most numerous planktonic organisms, responsible for transformation of net primary production (Tammert *et al*., 2005). In water column, the heterotrophic microorganism may be able to consume more than 60% of total primary production (Fuhrman and Azam, 1982) and play an essential role in degrade organic matter and transfer nutrients to food web via microbial loop (Azam *et al*., 1983), either directly releasing nutrients in surrounded water or indirectly being grazing by their predators. Also, their importance seems to increase as more oligotrophic the system is (Baines and Pace, 1991).

A central issue in ecology is to understand the factors involving environmental changes and the costs that these changes could promote in assemblages of organisms. Changes in the concentrations of key environmental factors lead to the differences in food web structure and in abundance of it major players (Tammert *et al*., 2005). Depending on organism`s perspective their distribution in environment may be shaped by multiple forces acting in complementary or antagonistic ways, but this heterogeneity may be orderly and reflect function and process or much of it may be simple randomness (Barker *et al*., 2010).

According to Allan (1995) microbial community can present a rapidly response to environmental changes. In lakes, these communities are temporal and spatial dynamics within and between habitats (Garland, 1997; Yannarell and Triplett, 2004), as a result of their interactions with other organisms, and the conditions and resources availability into the habitat.

Shallow lakes present high productivity due to be mainly governed by littoral zones and thus dominated by macrophytes, which control the primary production, rather than phytoplankton (Jeppensen *et al*., 1998; Moss, 1998). The structure and functioning of

shallow lakes rely mainly on these aquatic plants both in temperate lakes (Jeppensen *et al*., 1998; Scheffer, 1998) and, tropical and subtropical systems (Meerhoff *et al*., 2003), although, differences in climate zones in freshwater ecology had been documented (Meerhoff *et al*., 2007).

The accumulation of organic matter in bottom of shallow lakes exceeds its consumption (Danovaro and Pusceddu, 2007) There is a tendency regarding such lakes as exceptions but this is to underestimate their extent and importance for local and global biodiversity (Moss, 1998). Fluctuations in the water regime in shallow lakes tend to generate disturbances on water quality. Low water level can affect ecological functions of biological compartments as aquatic plants and phytoplankton (Finkler-Ferreira *et al.*, 2008) and thus may have an affect either in lower and upper trophic levels.

As microbial community develop such important role into aquatic ecosystem function, it would be useful to have an applicable technique to understanding microbial community functionality in water systems. Most studies involving microbial functioning in freshwater have been conducted in temperate lakes (Choi and Dobbs, 1999; Grover and Chrzanowski, 2000; Leflaive, *et al.*, 2008, Thottathil *et al*., 2008), whereas subtropical shallow lakes have only received negligible attention, mainly for composition and diversity studies (e.g., Haig-They *et al*., 2010; Rodrigues *et al.*, 2011; Canterle *et al*, unpublished data)**.** In this way patterns of carbon source utilization has been used over the past two decades as an useful and rapid tool to characterize heterotrophic microbial communities function in environmental samples (e.g., Garland and Mills, 1991; Choi and Dobbs, 1999; Grover and Chrzanowski, 2000; Goberna *et al.*, 2005; Leflaive, *et al.*, 2008; Tiquia, 2010).

In this study we investigated vertical and seasonal changes in heterotrophic microbial functionality following variation of environmental characteristic in a subtropical shallow

lake. So far, analysis of functional diversity in shallow lakes, using this approach remains poorly elucidated. Due to frequently disturbance events and mixture of water column in a shallow lake, seasonal variation was expected, whereas vertical changes were not so well estimated. The main goal of the study was to analyze functional stratification of heterotrophic bacteria between seasons. Also verify whether changes in microbial functionality were influenced by changes in the environment.

2. Materials and Methods

2.1. Study area and sampling

Lake Mangueira is located in Rio Grande do Sul, southern Brazil (~30°31'22"S 53°07'48"W). It is a large and coastal shallow lake, which comprises an area of 820km^2 (Fig.1). This ecosystem is mainly associated with wetlands and other lakes and is part of the Hydrological System of Taim and a Protected Area (Taim Ecological Station). This region represents a complex coastal freshwater system related to its biodiversity and its trophic dynamics (Finkler-Ferreira *et al.*, 2007).

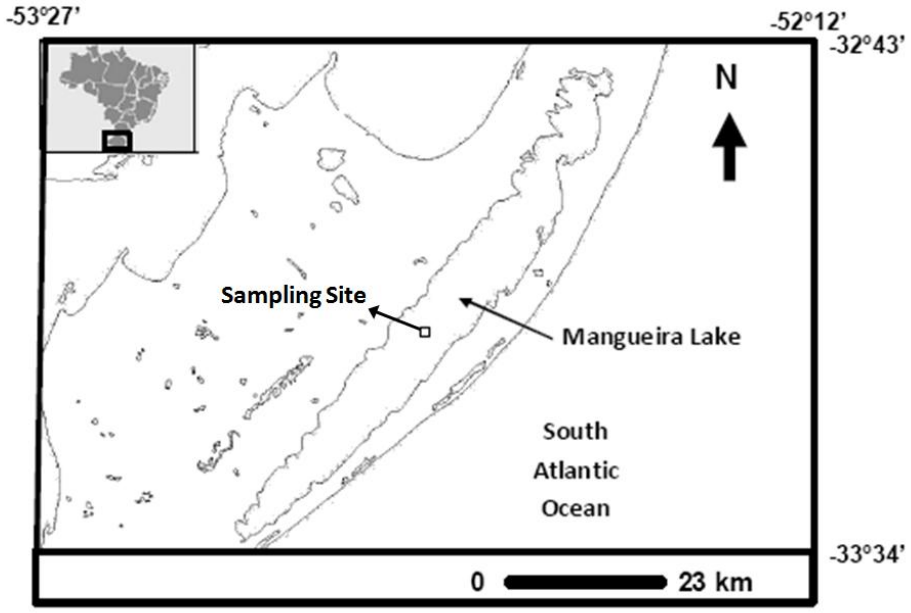


Fig. 1 Sampling area at Lake Mangueira, southern Brazil.

Samples were collected at every meter in a depth profile: in surface water, 1m, 2m, 3m, 4m and 5m; in the pelagic zone, in center of Lake Mangueira. Those samples were obtained during 2010 in summer (March), autumn (June), winter (end of July) and spring (November).

Environmental variables were gathered concurrently with heterotrophic microbial community samples. Turbidity (Turb), pH and water temperature were measured with a Multiparameter Water Quality Sonde. Secchi depth assessed with Secchi disk. Total Suspensed Solids (TSS) was assessed gravimetrically by water evaporation in porcelain dishes (APHA, 1999). Nutrients were evaluated through colorimetric methods: Total Nitrogen (TN), Nitrate (NO_3^-), and Soluble Reactive Phosphorus (PO_4^{-3}) following APHA (1999). Analysis of ammonium (NH_4^-) and Total Phosphorus (TP) followed Mackereth *et al*. (1989). Carbon Analyzer (Shimadzu Vcph 5000) was used to determine carbon series: Total Organic Carbon (TOC) was the unfiltered sample and the Dissolved Organic Carbon (DOC) was accounted as the fraction that passed through a 450 ºC pre-combusted glass

fiber filter (Macherey-Nagel GF6 - 0,6µm average mesh size). Chlorophyll *a* (Chla) was quantified after ethanol extraction (Wetzel and Likens, 1991).

The heterotrophic microbial community was sampled through 20µm mesh size sieve in 50 ml of amber glass bottle to avoid phytoplankton and detritus interference and then stored in the dark under refrigeration until analysis. Thus, Biolog Ecoplate^{TM} (Biolog, Inc. Hayward, CA) was used to access the community level physiological profile (CLPP) (Garland and Mills, 1991) of the community based on the carbon source they consume.

The approach of Biolog Ecoplates^{TM} is composed of three replicates of 31 ecologically applicable (Campbell *et al.*, 1997; Preston-Mafham *et al.*, 2002) carbon sources and a control. In each well, redox dye (tetrazolium dye) is added, which became purple as a result of formazan production, after carbon source was used by heterotrophic microorganism into the sample.

Samples from Lake Mangueira were inoculated as faster as possible after sampling and each well of Ecoplate, was directly inoculated with 150µl of sample and incubated at approximately 25^{o}C. The substrate utilization was assessed through optical density (OD) measured at 590 nm, with an automated plate reader (SpectraMax 5.0, Molecular Devices), immediately after incubation (t=0h) and every 24h during 12 days, to ensure that a saturation of the carbon utilization rate in all samples was reached (Salomo, 2009).

The community-level physiological profile (CLPP) was assessed by two different approaches using different data set. For data set analysis, triplicate substrate were averaged. An average well color development (AWCD) method (Garland and Mills, 1991) was applied, in which a kinetic profile was calculated (Lindstrom *et al.*, 1998), generating an overview of all heterotrophic metabolism microorganism. Additionally, the substrate diversity was assessed following the proposed by Zak *et al.* (1994) and the calculus theory

of Magurran (1988). The kinetic approach was used to overcome possible effects of inoculums density and time (Lindstrom *et al.*, 1998; Preston-Mafham *et al.*, 2002). Thus fitting the kinetic model to a density-dependent logistic growth equation based on a sigmoid curve; in this case the AWCD curve, two kinetic parameter (*K* and *r*) were used in this study because do not vary with inoculums density.

Furthermore, a more restrictive view was evaluated by curve integration (CI) method (Gukert *et al.*, 1996) which generated information of the 31 carbon source. The CI is a trapezoidal approximation and this approach incorporate additional information from the absorbance versus time incubation (lag phases, rates of color development and maximum absorbance) into a single number and is correlated with the kinetic parameters (Garland and Mills, 2001). The single value obtained was transformed in proportional substrate utilization efficiency (PSUE) to avoid effects of inoculums density (Pietikäinen, *et al*. 2000).

2.2. Data Analysis

In order to evaluate whether limnological variables and community level physiological profile (which include all different data set) were distinct during seasons and across depth profile, a *two-way* multivariate analysis of variance (MANOVA) with 999 permutation were performed, matching data matrices in time and space. As the CLPP is not comparable to species abundance, the analysis was based on Euclidian distance (Legendre and Legendre, 1998), used as a measured of resemblance among sites for both limnological and CLPP data, applied to transformed log (x+1) for limnological. The utilization of 31 carbon substrate was analyzed as PSUE. Also, for environmental data, an analysis of variance (ANOVA) was carried out between each variable, looking for what variable was responsible for mainly dissimilarities in each season. In both MANOVA and ANOVA

contrasts were used to find which groups were different from each other. Contrasts were as well evaluated by randomization testing (Pillar and Orlóci, 1996; Pillar, 2006).

To group samples according to their patterns of substrate utilization of each season, a hierarchical cluster analysis (HCA) was performed using Euclidian distance and group average linkage method. Further, a detrended correspondence analysis (DCA) using CI data was performed. This analysis established first gradient lengths less than 3.0 standard deviation units. This indicates that data should be subject to linear ordination methods (Ramette, 2007). Thus we used a principal component analysis (PCA), both for limnological and PSUE data of 31 carbon sources, to evidence temporal variation between samples, grouping the data by similarity and correlating the variables dynamic within each sample. For that, cross-product matrix was based on variance/covariance (Dufrêne and Legendre, 1997).

Additionally, was carried out an Indicator Species Analysis (Dufrêne and Legendre, 1997). The analysis intended to investigate substrates associated with each season. In the context of Biolog^{TM} the word indicator species analysis will be replaced with "indicator substrate analysis" as we are dealing with substrate utilization patterns as opposed to direct counts. The significance of the indicator value of each species was assessed by randomization procedure.

Finally, to evaluate if temporal variation of community level physiological profile was drove by changes in water characteristics, firstly Pearson product-moment correlations were evaluated between environment and functional attributes. Then the significant ($P \leq 0.05$) correlations of kinetic data were selected to be interpreted and the PSUE data was used to perform ordination analysis. Following the linear tendency of the data we used a redundancy analysis (RDA). One application of this analysis in microbial ecology is to

determine which environmental factors were the most significant to explain variation in microbial community (Ramette, 2007). It can be considerate as an extension of PCA, in which the main axes are constrained to be linear combinations of the environmental variables and multiple regression analyses are modeling a dependent variable using a set of exploratory variables (Rao, 1964; Legendre and Legendre, 1998).

We conducted the analysis of variance (ANOVA and MANOVA) using software Multiv v.2.4.2 (Pillar, 2006). HCA, PCA and RDA ordination and indicator substrate analysis were carried out in PCord 6.0 software (McCune and Mefford, 1999). The kinetic parameters for CLPP were calculated using the LabFit software (Silva and Silva, 2004).

3. Results

3.1. Environmental Variables

The limnological parameters of Lake Mangueira presented temporal (seasonal) variation (P=0.001) among the sampling sites. Vertical variation for this data was not significant (P>0.05) observed. For the PCA ordination (Fig. 2), the first two axes, accounted 97.65% of the total variance, 85.95% (P<0.0001) and 11.69% (P<0.002), respectively. Samples from autumn and summer were related with TOC (r=-0.80), DOC (r=0.-83), total solid suspensed (r=0.-82), chlorophyll *a* (r=-0.997), turbidity (r=-0.62), SRP (r=-0.83), negatively to axis 1. Samples from spring and winter were related positively to axis 1 and were not directly related with any variable. Along axis 2 warm seasons (summer and spring) were separated from cold season (autumn and winter).

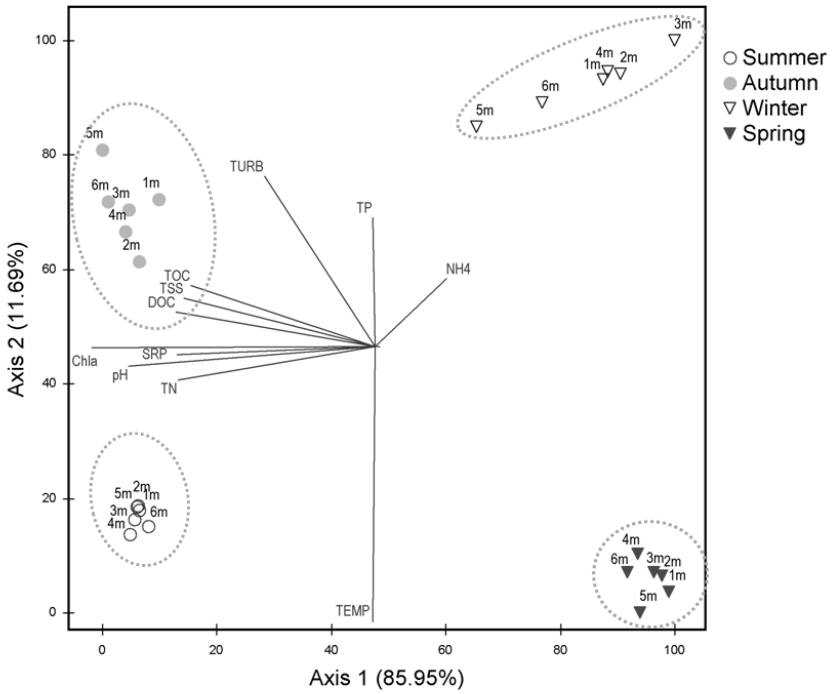


Fig. 2 PCA biplot ordination of limnological data sampled during summer, autumn, winter and spring.

Nutrients (total solid suspensed, total nitrogen and nitrate), chlorophyll *a* and temperature were higher on average concentration in summer, but some of those variables presented similar concentration, as showed summer, in other seasons, mainly during autumn (Table 1). Also during autumn, concentrations of carbon measured as TOC and DOC were higher, but very similar with summer. Comparable results observed above for summer and autumn, suggests that both season share similarities due to their proximity in time (i.e. one starts right after the other).

During winter, only ammonium presented high average concentrations. In spring, none limnological variables presented higher average concentration comparing to the other seasons.

Table 1. Limnological data (mean and standard deviation) of water samples from transversal profile at Lake Mangueira, during summer, autumn, winter and spring.

Season		TSS ($mg.l^{-1}$)	TP ($mg.l^{-1}$)	SRP ($mg.l^{-1}$)	TN ($mg.l^{-1}$)	NH_4^+ ($mg.l^{-1}$)	NO_3^- ($mg.l^{-1}$)	Chl*a* ($ug.l^{-1}$)	TOC ($mg.l^{-1}$)	DOC ($mg.l^{-1}$)	Temperature ^oC	Turbidity (NTU)	pH
Summer [a]	mean	12.17	0.03	0.02	0.64	0.05	0.16	42.83	19.33	18.03	22.57	5.33	8.34
	SD	1.69	0.00	0.00	0.10	0.03	0.10	1.83	1.25	1.90	0.05	0.50	0.02
Autumn [b]	mean	13.42	0.03	0.02	0.34	0.01	0.04	41.92	20.27	18.39	13.54	8.60	8.16
	SD	2.99	0.01	0.00	0.03	0.00	0.00	3.49	1.18	0.55	0.00	0.54	0.00
Winter [c]	mean	9.42	0.03	0.01	0.13	0.19	0.11	4.27	17.42	15.48	11.42	6.48	7.86
	SD	0.58	0.00	0.00	0.03	0.07	0.02	1.97	1.48	0.64	0.16	0.21	0.01
Spring [d]	mean	6.00	0.03	0.01	0.15	0.05	0.10	3.80	13.29	12.68	21.77	2.65	7.88
	SD	0.63	0.00	0.00	0.02	0.03	0.03	0.39	0.37	0.50	0.04	0.16	0.01
ANOVA P-*values*		0.001***	0.008**	0.001***	0.001***	0.001***	0.013*	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
Summer vs. Autumn		0.453^{NS}	0.079^{NS}	0.816^{NS}	0.003**	0.005**	0.063^{NS}	0.619^{NS}	0.22^{NS}	0.62^{NS}	0.006**	0.005**	0.006**
Summer vs. Winter		0.015*	0.006**	0.005**	0.004**	0.003**	0.292^{NS}	0.004**	0.029*	0.003**	0.003**	0.005**	0.002**
Summer vs. Spring		0.007**	0.255^{NS}	0.004**	0.002**	0.766^{NS}	0.295^{NS}	0.002**	0.002**	0.001***	0.001***	0.002**	0.006**
Autumn vs. Winter		0.017*	0.808^{NS}	0.003**	0.003**	0.005**	0.001***	0.001***	0.011*	0.006**	0.003**	0.003**	0.002**
Autumn vs. Spring		0.006**	0.035*	0.003**	0.001***	0.002**	0.002**	0.006**	0.004**	0.004**	0.003**	0.004**	0.003**
Winter vs. Spring		0.002**	0.003**	0.207^{NS}	0.24^{NS}	0.003**	0.766^{NS}	0.797^{NS}	0.003**	0.004**	0.002**	0.001***	0.034*

TSS, total suspensed solids; TP, total phosphorus; SRP, soluble reactive phosphorus; TN, total nitrogen; Chl *a*, chlorophyll *a;* TOC, total organic carbon; DOC, dissolved organic carbon; NTU, nephelometric turbid unit; vs., versus. Mean values (n=6). *MANOVA*, different letters indicates significantly variation P≤0.05. *ANOVA* *P≤0.05; **P≤0.01;***P≤0.001; ns= not significant.

3.2. Community Level Physiological Profile

The overall heterotrophic microbial metabolism assessed by the average well color development (AWCD) presented a sigmoid curve course as typical expected, for all season (Fig. 3). This curve represents important growing phases of microorganism: a lag phase in the beginning, first 48h, which means that the community is adapting to the new condition that it was exposed after incubation; an exponential phase, when the growing cells presented a good physiological condition in the culture, and finally, the asymptote, when bacterial cells after reached the carrying capacity, start to die.

Those AWCD curves were the base to compare, in terms of kinetic approach, the temporal variation of samples from the vertical profile at Lake Mangueira, for overall metabolism.

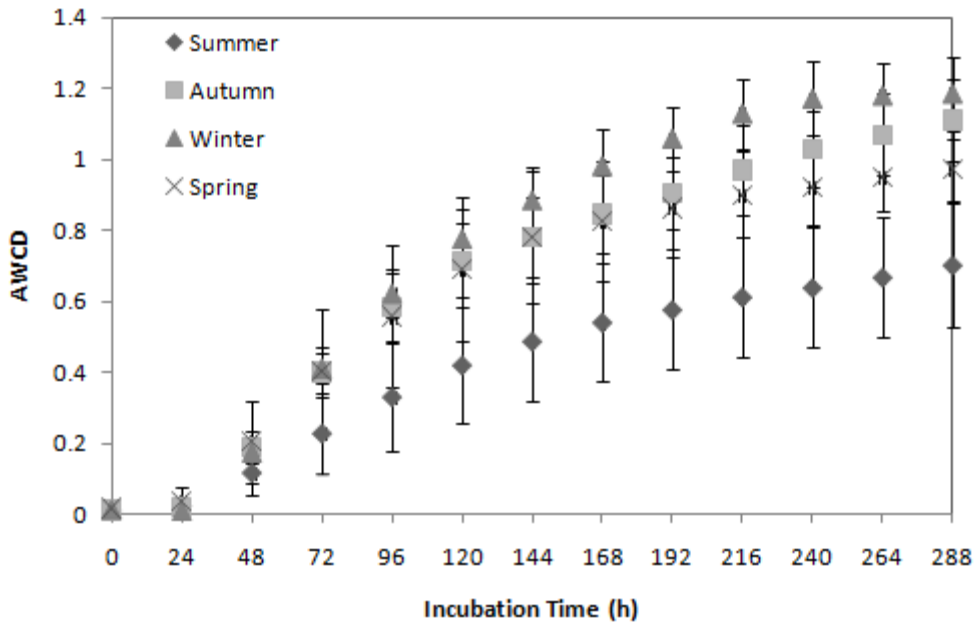


Fig. 3 Time course curve of AWCD in summer, autumnm winter and spring at Lake Mangueira. Means and standad deviation are displayed (n=6).

The analysis of the kinetic parameters fitting the AWCD curves to the density-dependent logistic growth showed significant ($P \leq 0.05$) temporal differences only for the *K*

parameter over the seasons, except between autumn and winter which were similar (P>0.05) (Table 2). The *K* parameter represents the asymptote of the curve, which means the maximum degree of substrate utilization, i.e. considering that *K* parameter could be interpreted as carrying capacity; each season reached different carrying capacities in terms of AWCD. Cold seasons presented the higher average value of *K* and, in contrast, spring and summer (warm season) presented lowest values, respectively.

Table 2. Kinetic parameter, *K* and *r* from fitting the AWCD curve to the density logistic growth equation.

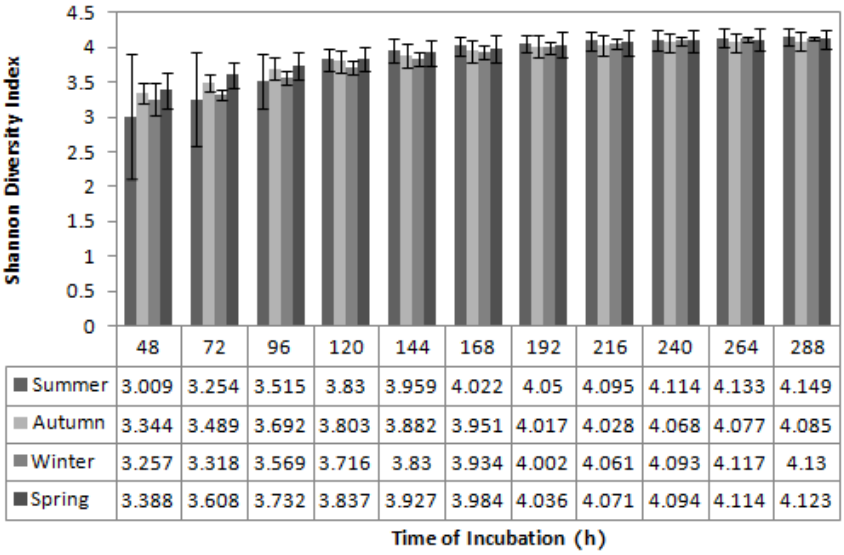
Season	K		r	
	Mean	SD	Mean	SD
Summer	0.66 ±	0.005 [a]	-0.03 ±	0.000 [a]
Autumn	1.04 ±	0.010 [b]	-0.03 ±	0.001 [a]
Winter	1.15 ±	0.003 [b]	-0.03 ±	0.000 [a]
Spring	0.94 ±	0.005 [c]	-0.04 ±	0.001 [a]

Signal of minus, population is decreasing. Different letter indicates significant variation. Mean values (n=6). ANOVA P<0.05.

On the other hand, *r* parameter that represents the velocity of substrate utilization did not vary among the seasons. The variability of *K* may represent more availability of specific resources during autumn and winter, rather than in summer and spring. Also the lack of variability of *r* parameter indicates that the rate of net increase in population became the same across the season. Ultimately, none of kinetic parameters presented significant (P>0.05) vertical variation.

The functional diversity assessed by Shannon diversity index (H') include both substrate richness and substrate evenness and represents the ratio of activity of a particular substrate to the sum of activity of all substrates. For this approach, the H' ranged on average from 3.09 in summer, in the beginning of incubation time, to 4.15 at the end of incubation also in summer. Despite slightly variation across the seasons and sites, none

temporal and spatial variation was significant in Lake Mangueira (Fig. 4). In this way, for all seasons, the heterotrophic metabolism activity appears to be very homogeneous.



	48	72	96	120	144	168	192	216	240	264	288
Summer	3.009	3.254	3.515	3.83	3.959	4.022	4.05	4.095	4.114	4.133	4.149
Autumn	3.344	3.489	3.692	3.803	3.882	3.951	4.017	4.028	4.068	4.077	4.085
Winter	3.257	3.318	3.569	3.716	3.83	3.934	4.002	4.061	4.093	4.117	4.13
Spring	3.388	3.608	3.732	3.837	3.927	3.984	4.036	4.071	4.094	4.114	4.123

Fig. 4 Shannon diversity index of heterotrophic microbial metabolism derived from carbon substrate utilization along 288h of incubation during summer, autumn, winter and spring. Means and standard deviation are displayed (n=6).

The utilization pattern of the 31 carbon substrates by bacterial assemblage were significantly different between season (P=0.001). The cluster analysis formed four mainly groups, and thus, samples of each season were grouped together. Consequently, became evident that the temporality was very strong ruling events over all season (Fig.5). Still, a bigger group with similarity around 55% connected samples from autumn, winter and spring, and another group with approximately 87% of similarity linked samples from cold seasons as autumn and winter.

Despite no differences within the vertical gradient were evident, and during winter, all samples displayed 100% of similarity, and in the other seasons the similarity between sites was above 95%. Samples from summer and spring appeared to be more dissimilar than samples from winter and autumn.

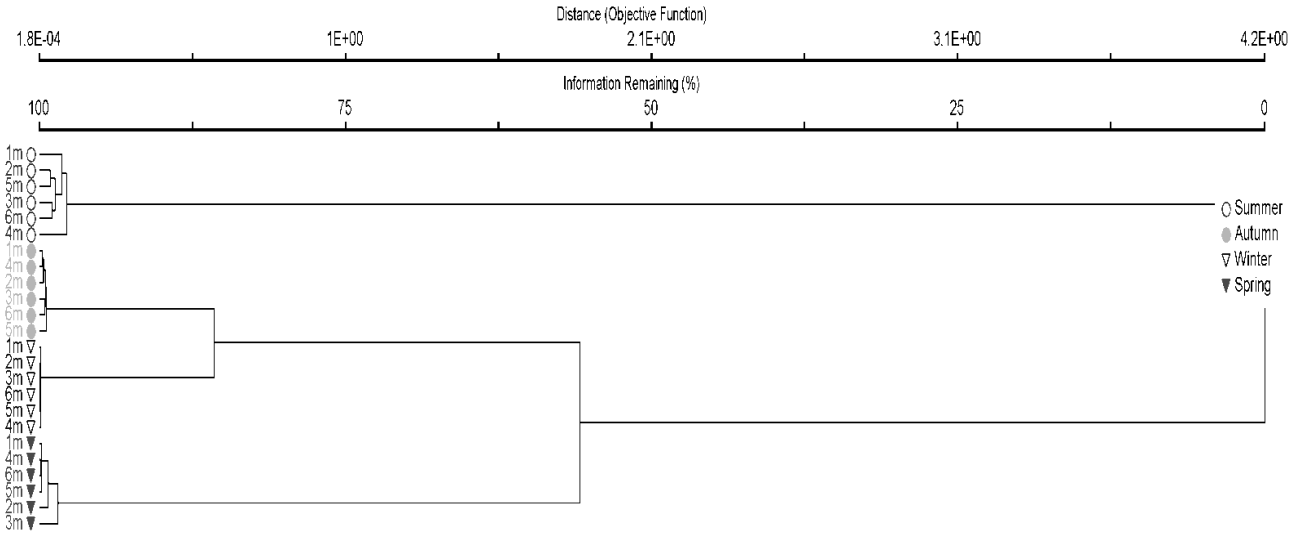


Fig. 5 Cluster analysis of proportional substrate utilization efficiency data, from samples collected during summer, autumn, winter and spring at Lake Mangueira.

The PCA ordination (Fig. 6) showed significant ($P<0.05$) variation along axis 1, which explained 35.06% of variation. The axis 2 explained 14.07% of variation. Although there were a total of many PCA axes as there were substrates (n=31), the first two components were retained for interpretation following the broken stick model (Jackson, 1993; McCune and Mefford, 1999). Samples from summer were positively correlated to axis 1 and appear to be more disperse than the sample from other seasons that were negatively correlated to this axis. The carbon substrates represented by the codes E2, H1 and G1 are carbohydrates; D1 is a polymer and H4 is an amine and they are well positively correlated along axis 1 with samples from summer. On the other hand, negatively correlated to axis 1 were found a mix of different groups of substrate characterizing sample from autumn, winter and spring.

The Indicator Substrate Analysis revealed that those substrates well positively correlated to axis 1 of PCA plus other two carbon sources were significantly ($P\leq0.05$) associated with summer (Table 3): amine – Putrescine (H4); carbohydrates – α-D lactose (H1); D-cellobiose (G1); D,L-α-glycerol phosphate (H2); N-acetyl-D-glucosamine (E2);

polymers – Tween 80 (D1), Glycogen (F1). According to the analysis, three carbon sources, strongly negative correlated with axis 2 ($r>0.51$; $P<0.05$), were associated with samples from autumn: D-glucosaminic acid (F2) and D-Malic acid (H3), both belonging to carboxylic acids substrate groups; and α-cyclodextrin, a polymer. In winter, six substrates were associated to this season: an amine – Phenylethylamine (G4); an amino acid – L-Phenylalanine (C4); two carbohydrates – I-Erythritol (C2) and D-Xylose (B2); a carboxylic acid – Itaconic acid (F3); and a Phenolic Compound – 2-Hydroxy benzoic acid (C3). Two substrates associated with winter did not show any significant correlation with axis 2 of PCA, where samples from winter were associated. In addition, none carbon substrates were significantly associated with samples from spring.

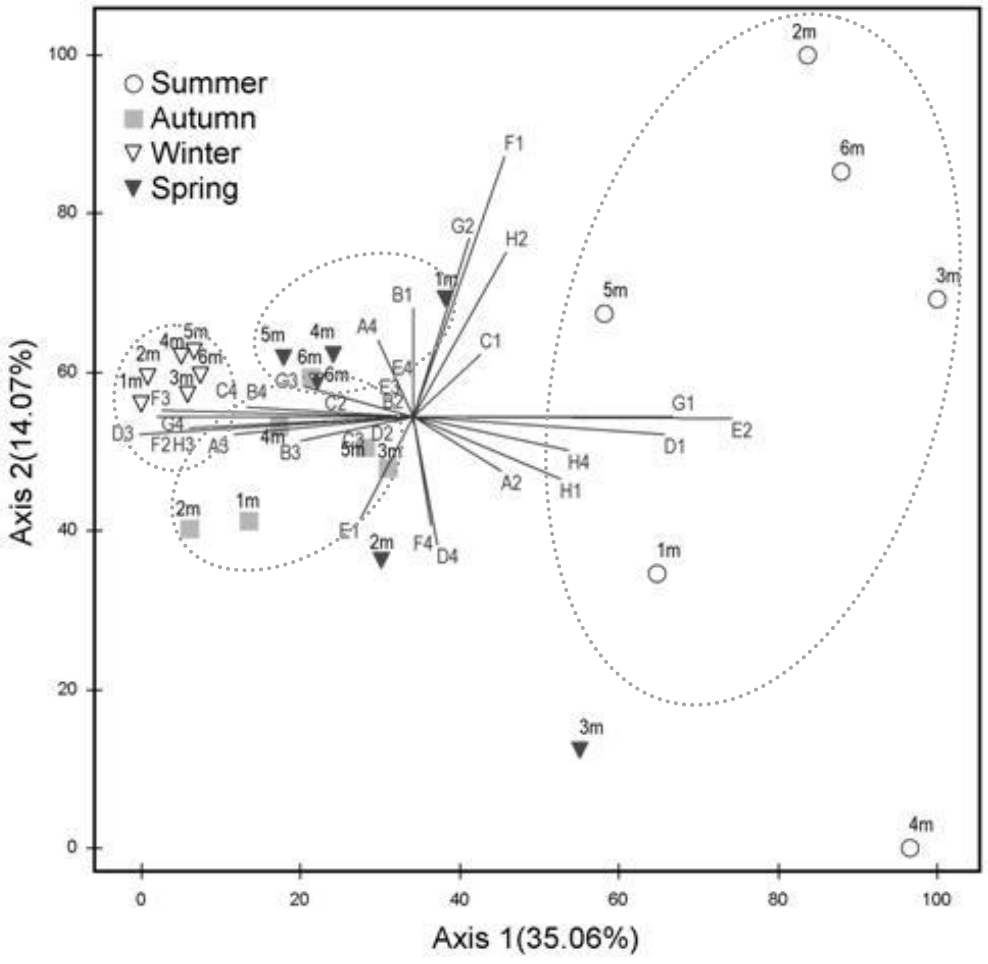


Fig. 6 PCA biplot ordination diagram of proportional substrate utilization efficiency samples from Lake Mangueira, collected during summer, autumn, winter and spring.

Table 3. Main results of correlation coefficients for scored of the first two principal component analysis (PCA), of indicator substrate analysis and of *two-way* MANOVA from efficiency of substrate consumption data in each season at Lake Mangueira.

Carbon Sources		Overall Season				
		Correlation		Indicator Substrate		
	Code	Axis 1	Axis 2	Season	% of perfect indication	*P-values*
Amines/amides						
Phenylethylamine	G4	-0.71		winter	53.7	0.001***
Putrescine	H4	0.63		summer	41.1	0.03*
Amino acids						
L-Arginine	A4		0.44			
L-Asparagine	B4	-0.60				
L-Phenylalanine	C4	-0.65		winter	36.3	0.049*
L-Serine	D4		-0.57			
Glycyl-L-glutamic acid	F4		-0.52			
L-Threonine	E4					
Carbohydrates						
α-D-lactose	H1	0.61		summer	37.7	0.031*
β-Methyl-D-glucoside	A2	0.47				
D-Cellobiose	G1	0.81		summer	34.5	0.02*
D-Mannitol	D2					
I-Erythritol	C2	-0.41		winter	38.1	0.013*
Glucose-1-phosphate	G2		0.67			
D-Galactonic acid -Υ-lactone	A3	-0.67				
N-Acetyl-D-glucosamine	E2	0.90		summer	36.7	0.002**
D,L-α-glycerol phosphate	H2	0.48	0.65	summer	44.9	0.04*
D-Xylose	B2			winter	41.9	0.002**
Carboxylic & acetic acids						
α-ketobutyric acid	G3	-0.52				
D-Galacturonic acid	B3	-0.53				
D-Glucosaminic acid	F2	-0.80		autumn	35.1	0.008**
Υ-Hydroxybutyric acid	E3					
D-Malic acid	H3	-0.75		autumn	35.8	0.043*
Pyruvic acid methyl ester	B1		0.52			
Itaconic acid	F3	-0.80		winter	42.3	0.036*
Polymers						
α-cyclodextrin	E1		-0.51	autumn	33	0.038*
Glycogen	F1	0.48	0.81	summer	37.8	0.015*
Tween 40	C1	0.41				
Tween 80	D1	0.80		summer	35.5	0.003**
Phenolic Compounds						
2-Hydroxy benzoic acid	C3			winter	64.8	0.001***
4-Hydroxy benzoic acid	D3	-0.83				
MANOVA	P-*values*:					
Season		0.001***				
Summer vs. Autumn		0.02*				
Summer vs. Winter		0.034*				
Summer vs. Spring		0.029*				
Autumn vs. Winter		0.019*				
Autumn vs. Spring		0.47^{ns}				
Winter vs. Spring		0.036*				

r value>0.41 are significant (P≤0.05). Indicator substrate analysis and MANOVA *P≤0.05; **P≤0.01; ***P≤0.001; ns, not significant (n=24).

The Pearson product-moment correlation revealed that the kinetic parameters presented correlation with some environmental variables (Table 4). *K* parameter showed strong, and significantly (P≤0.05) negative correlation with TN, water temperature, pH and Chl*a*. These were the mainly environmental variables well related to samples from summer, what may explain higher value of this parameter among cold season as winter and autumn. However, *r* parameter, which was very similar among all season; presented significant negatively correlation with several variables.

Table 4. Pearson product-moment correlation between Kinetic parameters and environmental variables.

	K	*r*
TSS	-0.16	**-0.62**
TP	0.37	-0.15
SRP	-0.34	-0.28
TN	**-0.78**	**-0.43**
NH_4	0.32	-0.04
NO_3	-0.26	0.11
CHL*a*	**-0.45**	**-0.45**
TOC	-0.08	**-0.55**
DOC	-0.24	**-0.57**
TEMP	**-0.74**	0.21
TURB	0.30	**-0.49**
pH	**-0.65**	**-0.43**

Significant (P≤0.05) values are in bold.

The RDA ordination significantly (P=0.001) explained 44.4% of total variation of the samples (Fig. 7). RDA1 and RDA2 accounted for 34.9% and 9.4% respectively. According to this analysis, axis 1 significantly (P=0.001) presented Pearson correlation between response and predicts variables of r=0.97. It was evident how samples from summer were more disperse than from other season. Most nutrients (nitrate, total nitrogen, SRP), chlorophyll and temperature created different responses condition along the samples

from summer, positively correlated to axis 1; thus, such situation shaped the microbial assemblage to explore different kind of carbon source. In this way, five carbon substrates, H4 (r=0.51), H1 (r=0.56), G1 (r=0.78), F1 (r=0.57) and D1 (r=0.74), identified previously as indicative of summer, were straightly related to TN(r=0.80), Chl*a* (r=0.50), water temperature (r=0.77), SRP (r=0.45) and NO_3 (r=0.57) along axis 1. Although the seasonality was identified, the majority samples from autumn, and spring were ordinate very nearly among each other and none gradient of functional attributes being positively correlated with environment variables became evident.

In samples from winter, conditions of NH_4 (r=-0.33) and TP (r=-0.41) influenced changes in the microbial community to explore different available substrates as amine, G4 (r=-0.69), amino acid, C4 (r=-0.63), carbohydrates, C2 (r=-0.43) and B2 (r=-0.21) and carboxylic acid, F3 (r=-0.79). Such conditions shaped the pattern of strong similarity among samples in winter.

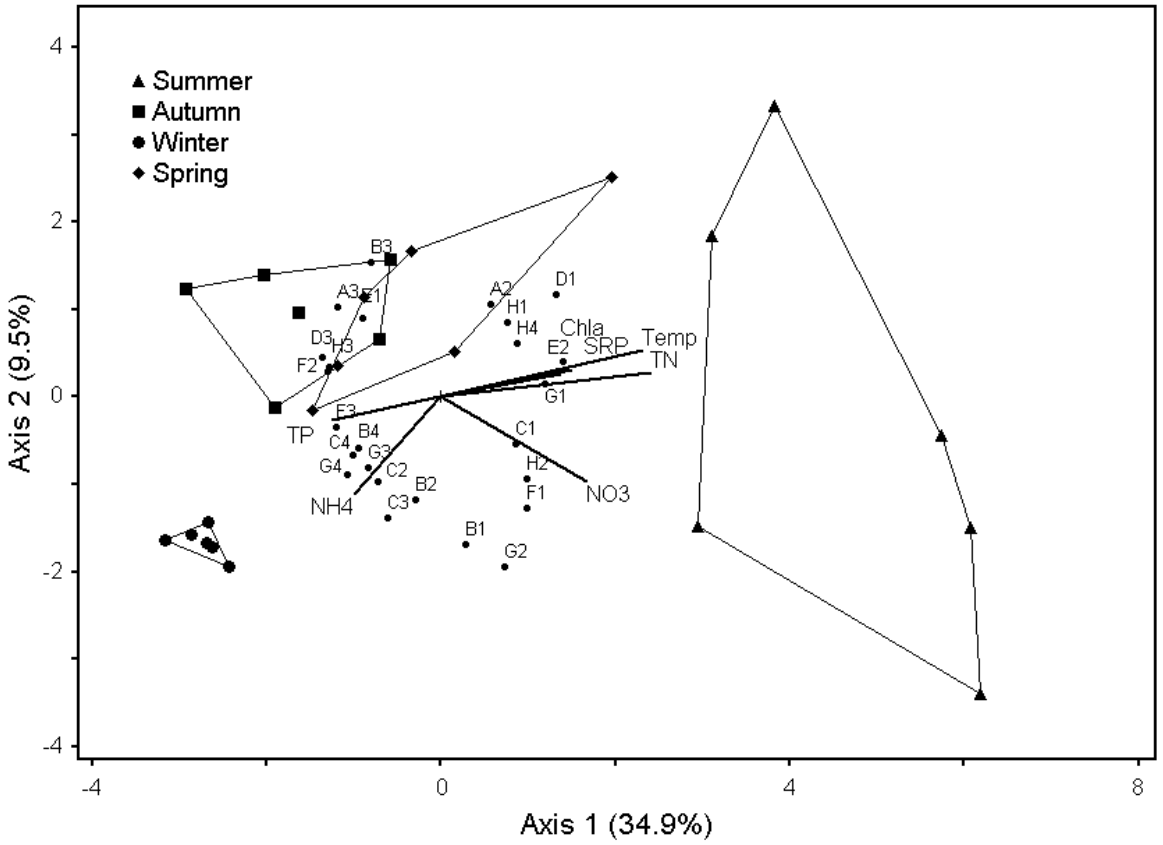


Fig. 7 Redundancy Analysis triplot diagram of environmental variables PSUE data, during summer, autumn, winter and spring at Lake Mangueira. The name of the carbon source are displayed in table 3.

4. Discussion

Notable seasonal variability was identified across the deep profile in water characteristics and among heterotrophic bacteria assemblage function in the pelagic zone at Lake Mangueira. Consistent seasonal pattern in substrate utilization by bacterioplankton were also found in different temperate lakes and other aquatic environments (Grover and Chrzanowski, 2000; Christian and Lind, 2007, Bradley and Owen, 2007). The bacterioplankton assembly may functionally and compositionally reflect high sensitive and comprehensive responses to the whole lake conditions (Tian, *et al.*, 2009; Haig-They *et al.*, 2010), explaining the variability found across the seasons. In this ways, shifts in primary production rates, changes in dissolved organic matter nature or variations in grazing rate, are one of the major sources of short-term variability of aquatic bacteria community (Santos *et al.*, 2009). Moreover, seasonal cycles of environmental parameters in Lake Mangueira are key factors driving the planktonic communities (Rodrigues *et al.*, 2009), and thus driving its metabolic diversity.

In this study, the carbon substrate utilization varied in association with season condition. Consequently, during summer, more substrates were significantly utilized, compared to the other season. The carbon substrates mostly consumed, in summer, were carbohydrates in association with warm temperatures, higher chlorophyll *a*, nutrients, TOC and DOC. In a less proportion also were utilized amine and polymers. The high carbohydrate utilization may indicate that the bacteria populations that are opportunistic and capable of using carbohydrates increase when the supply of carbohydrates is increased (Tiquia, 2010). Also, high relative responses to carbohydrates in Biolog, may be coincided to a peak of algal abundance in late summer, as found by Grover and Chrzanowski (2000) in temperate lakes and likely due to the carbon fixed by

phytoplankton that is released as dissolved organic matter, and much of this correspond to carbohydrates (Biddanda and Benner, 1997).

During autumn bacterial assemblages preferably used carboxylic acid and polymer over carbohydrates and amine. Carboxilic acid are often naturally a product of bacterial fatty acid catabolism, photochemical degraded (Bertilsson and Tranvik, 2000). Probably the higher preference of this substrate is due to the increased amount of organic acid that happened during summer by longer photoperiods selecting bacteria that are able to utilize organic acids (Christian and Lind, 2007) but only influenced the assemblage in autumn.

During the onset of winter, several substrates belonging to a variability of guilds of substrates were exploited. Nonetheless, only one substrate was utilized in each guild; except for carbohydrates, which two substrates were utilized. Also none polymers were consumed. In the RDA ordination (Fig. 7), the utilization of such substrates was associated to TP and NH_4. The pattern substrate utilization may be expressing high plasticity in metabolic pathways (Sala *et al*., 2006) related to the variety of guilds of substrates, in which bacteria assemblage are opportunistic to growth in different groups. Or may be reflecting many specialists broadly distributed among taxonomic groups (Colwell and Lehman, 1996).

In contrast, during spring, when none limnological variable were positively correlated to samples from this season, none substrate demonstrate significant associated to this season by the substrate indicator analysis. According to Tiquia (2010), a slightly more substrates were utilized in summer than in spring in Rouge River, likely due to the higher bacterial density and lower DO of water samples in summer. Despite these variables were not measured for this study, and the study area be different from our Lake this could be an explanation for the trend observed.

Considering that nutrient and temperature limit bacterioplankton production (Morris and Lewis, 1992; Schweitzer and Simon, 1995; Vrede, 2005), such limitation may affect both shape and size of bacteria thereby influencing grazing patterns on it and thus affecting the bacterial community structure (Vrede, 2005), which consequently may reflect different pattern of substrate utilization according to their capability to use different substrates over the season.

Even though overall metabolism and the utilization of each substrate demonstrate significant variation among seasons, the substrate diversity, measured as the ratio activity on a particular substrate to the sum of activities on all substrates (Zak, *et al*., 1994) did not presented such variability. According to Ramette (2007), because diversity index pool the multispecies information into a single value, for each observation, it is not surprising that complex diversity patterns may not be identified sometimes.

As expected, no vertical pattern was identified in this study. Coastal shallow lakes, as Lake Mangueira, are much influenced by wind, whether they are temperate, subtropical or tropical (Cardoso and Motta Marques, 2009), and it generates fluctuations and turbulence in the water column, providing lot of energy to suspend fine sediments. According to Motta Marques (2003) aquatic macrophytes could have an important role reducing ressuspension by easing wind action, but this relationship may not be a rule, because macrophytes beds seems to reduce their capacity under higher water levels associated to wind velocity. At Lake Mangueira, the samplings were performed in the pelagic zone, right in the center of Lake, reflecting higher water levels and higher wind velocity, which fetch can reach 90km/h, predominant from NE to SW (Fragoso Jr. *et al*., 2008) and so creating condition of turbulence in the pelagic zone, preventing stratification events.

Still, despite it was not observed significant depth variation across microbial community function within season in Lake Mangueira, summer samples showed more disperse than the others. Probably a slightly stratification was detected by the microbial community perspective, instead by our analysis, in association with limnological lake condition shaping the functionality of these microorganisms. According to Davey *et al*. (2001), stratified bacterial populations may determine patterns of organic matter hydrolysis in the water column. Free-living cells of bacteria may dominate pelagic zone and influence the degradation of organic matter in different ways through specific enzymes (Wetzel, 1992; Rehnstam *et al*., 1993; Martinez *et al*., 1996; Madigan *et al*., 2004).

Even though Biolog Ecolog^{TM} assay may be a selective method, the presence of rare members might be detected (Haack *et al*., 1995). Also, although this assay may not reflect the overall in situ function microbial community, this rapid technique remains a valuable tool for comparison of microbial communities function (Smalla *et al*., 1998) which produces a rich data set that is ideal for detecting site-specific differences in the functional diversity of microorganism (Zak *et al*., 1994).

Finally, we were able to identify pattern that corroborated our mainly hypothesis. Thus functional potential of the bacterial assemblages varied among season as a result of environmental lake characteristics playing a role in constrain the community among season. Also the weak stratification of the functional attributes, detected during summer and spring, is an indication of lake stratification not clearly indicated by temperature and proxies such as Chl *a*, TSS and Turbidity.

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6 Considerações Finais

A investigação da dinâmica funcional da comunidade microbiana aquática foi aplicada na Lagoa Mangueira, extremo sul do Brasil, pela primeira vez e, até o momento, esse tipo de análise ainda não havia sido contemplada para sistemas subtropicais, ficando restrita a sistemas de clima temperados.

Acreditamos que foi possível alcançar os objetivos propostos, considerando: (i) o potencial funcional microbiano foi acessado através das técnicas do consumo de substratos de carbono disponíveis; (ii) na evolução deste estudo, foi possível identificar a heterogeneidade funcional da comunidade microbiana, no sentido em que as características intrínsecas da lagoa interagem com a comunidade, contribuindo para a variação temporal e espacial de preferências ou habilidades no consumo de substratos de carbono; e (iii) o trabalho de investigação da diversidade funcional e de padrões relacionados às capacidades de utilização de substratos pela comunidade microbiana heterotrófica aquática foi viabilizado com a utilização do sistema Biolog Ecoplate ™. Essa abordagem permitiu avaliar a ocorrência de mudanças na comunidade microbiana em relação aos seus atributos funcionais.

A utilização deste método vem sendo discutida por pesquisadores de distintas subáreas relacionadas à ecologia microbiana, pois essa abordagem requer cautela, uma vez que confusões acerca da interpretação dos resultados podem ocorrer. Por exemplo, esse método não permite a determinação da composição específica microbiana de amostras naturais, mas como apresentado aqui, é muito útil para caracterizar e diferenciar atributos funcionais dessas comunidades e, de forma complementar, relacionar essas características em um contexto ecológico.

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