Universidade Federal do Rio Grande - FURG

Instituto de Ciências Biológicas (ICB)

Programa de Pós-Graduação em Ciências Fisiológicas -

Fisiologia Animal Comparada

IMPACTO DE AGENTES ESTRESSORES EM COMUNIDADES BACTERIANAS: DINÂMICA E INTERAÇÃO DE ESPÉCIES EM AMBIENTES HETEROGENEOS AFETADOS POR CONTAMINAÇÃO POR COBRE

Bióloga Rafaela Elias Letts

Tese submetida ao Programa de Pós-Graduação em Ciências Fisiológicas – Fisiologia Animal Comparada, como parte dos requisitos para a obtenção do título de DOUTOR em Fisiologia Animal Comparada.

Orientador: Dr. José M. Monserrat

Rio Grande

2012

Sumário

AGRADECIMIENTOS

A mi orientador, Prof. Dr. José María Monserrat, por el tiempo y espacio ofrecidos para desarrollar esta tesis.

A los profesores que aceptaron componer el jurado de evaluación de esta tesis.
Dr. Luis Fernando Marins, Dr. Luiz Eduardo Nery y Dr. Paulo Abreu.

A FINCyT por la beca otorgada para el desarrollo del doctorado.

Al Programa de Pós-graduación en Ciencias Fisiológicas – Fisiología Animal Comparada por la oportunidad de adquirir conocimientos en esta área de la ciencia.
En especial a los docente, por las disciplinas impartidas.

A los queridos colegas y amigos gracias por la compañía y ayuda en los experimentos: Josencler, Lygia, Flavio, Liani, Vinicius, Lilian (los de la sala 3); Jaqueline Kautz, Danielle y tia sillllva (las de Casino); Carla, Paola, Silvia y Diego (los de Lima) y a la Familia Monserrat (Malena, Laura y José).

Finalmente a mi madre, María Rafaela Letts Colmenares por absolutamente ¡TODO!.

RESUMO

Os manguezais são ecossistemas extremamente singulares, onde a composição e integridade dos bosques destes sistemas possuem um papel importante no desenvolvimento dos ciclos biológicos. As folhas que caem constantemente dos árvores dos manguezais aos estuários como mecanismo de eliminação de sais pressentes na água, é uma fonte importante de matéria orgânica assimilável. Por outro lado, a distribuição e composição das comunidades que conformam este ecossistema estão governadas por vários fatores como mudanças latitudinais (incluindo temperatura e precipitações), geomorfologia, substrato, salinidade, fluxos de alagamento e a topografia do terreno. Estas características fazem deste tipo de ecossistema um ambiente particular no qual os diversos organismos que o habitam tem desenvolvido mecanismos para se adaptar. No Perú, os manguezais de Tumbes (-3.57 LS e -80.44 LW UTM) estão seriamente impactados como resultado do desenvolvimento urbano, a indústria de pesca, a construção de canais artificiais, resíduos agroquímicos, metais pesados e a desmatamento indiscriminada de árvores. Este tipo variado de fatores perturbadores podem induzir a geração de estresse oxidativo e, por outro lado, a diversidade de bactérias é seriamente afetada por impactos mecânicos como a geração de canais artificiais que alteram a composição do consórcio bacteriano e a distribuição de contaminantes. Devido a alta produtividade gerada pela abundância de nutrientes como parte do ciclo biológico dos manguezais, as bactérias são um dos grupos de organismos mais importantes, cumprindo um papel fundamental na decomposição da matéria orgânica e nos processos geoquímicos. Muitas destas bactérias se encontram formando consórcios com outros organismos como diatomáceas, gerando estruturas multilaminares embebidas em uma matriz polimérica que se estende sobre a superfície

do substrato. Esta associação caracteriza-se por apresentar uma intrincada cadeia de associações funcionais conhecidas como biofilmes. Avaliar a estrutura funcional e a tolerância dos biofilmes frente a fatores de estresse relevantes neste ecossistema como a radiação UV, metais pesados e fatores ambientais como temperatura, pH, salinidade e nutrientes, permitiria ter uma aproximação das possíveis vias de tolerância e níveis de resposta destes consórcios. Por tal motivo, foi proposto avaliar a diversidade de bactérias dos biofilmes e sua respectiva capacidade antioxidante como indicador do estresse gerado e sua capacidade de resposta frente aos fatores avaliados. Os resultados mostram uma mudança constante dos consórcios bacterianos, observando-se uma melhor tolerância em aqueles consórcios que vem de águas hipersalinas. A diversidade e dinâmica dos consórcios bacterianos mostram um agrupamento dependendo do fator ambiental, sendo o cobre como um dos fatores que geram maiores alterações da composição original.

Palavras chave: manguezais, contaminação, estresse oxidativo, comunidades bacterianas

ABSTRACT

Mangroves are extremely important ecosystems, where the composition and integrity of the forests of these systems have an important role in the development of biological cycles. The leaves that constantly fall of mangrove trees on the estuaries as a mechanism for removing accumulated salts, this process making organic matter available. On the other hand, distribution and composition of the communities that make up this ecosystem are governed by several factors like latitudinal changes (including temperature and precipitation), geomorphology, substrate, salinity, stream flooding and topography of the land. These characteristics make this kind of ecosystems a particular environment in which the various organisms that inhabit it have developed several adaptation mechanisms. In Peru, the mangroves of Tumbes (-3.57 LS and -80.44 LW UTM) are seriously impacted as a result of urban development, the fishing industry, the construction of artificial canals, waste pesticides, heavy metals and indiscriminate splint trees. This type of varying disturbing factors may induce the generation of oxidative stress and, on the other hand, the diversity of bacteria is seriously affected by mechanical impact such as artificial channel generation altering the bacterial consortium composition and the distribution of contaminants. Due to the high productivity generated by the abundance of nutrients as part of the life cycle of mangroves, bacteria are one of the most important groups of organisms, fulfilling a key role in the decomposition of organic matter and geochemical processes. Many of these bacteria are forming consortia with other organisms such as diatoms, generating multilayer structures embedded in a polymeric matrix which extends over the substrate surface. This association, with an intricate chain of functional associations, is known as biofilm. Assessing the functional structure of biofilms and tolerance against stress factors such

as UV radiation, heavy metals, temperature, pH, salinity and nutrients, would generate information about the possible pathways of tolerance and response levels of these consortia. For this reason it was proposed to evaluate the diversity of bacteria that form these biofilms and their respective antioxidant capacity as an indicator of stress and their capacity to respond to the factors evaluated. The results showed a constant change of bacterial consortia, showing a better tolerance those consortia that comes from hyper saline brines. The diversity and dynamics of bacterial consortia showed a group depending on the environmental factors and copper as one of the factors that generate larger changes in the original community composition.

Key words: wetlands, pollution, oxidative stress, bacterial communities

1. INTRODUÇÃO

Nas últimas décadas, as teorias ecológicas tem mudado seu enfoque a respeito de assumida a homogeneidade do ecossistema, reconhecendo a heterogeneidade como um fator chave para entender a complexidade da natureza (Wiens 1989, Levin 1992). No entanto, o papel dos organismos nas mudanças da estrutura espacial a diferentes escalas, é ainda um campo que requer maior estudo. Deve se considerar que a relação entre escala e estrutura é muito estreita, apresentando-se em células até paisagens, incluindo a distribuição e abundância dos organismos dentro de um ecossistema (Hutchinson 1953, Turner 2005). Forman e Godron (1981) descreveram os ecossistemas heterogêneos como unidades ecológicas, onde as interações abióticas e bióticas ocorrem num mosaico de remendos que mudam sua estrutura e funcionamento através do tempo. O entendimento dos mecanismos que estão por trás da formação da estrutura espacial é essencial para compreender o funcionamento dos ecossistemas desde sua escala mínima até sua dimensão global (Gustafson 1998, Green e Sadedin 2005, Turner 2005, Battin *et al*. 2007).

Diversos estudos têm proposto que o efeito positivo entre os organismos e seu meio ambiente podem induzir estados de estabilidade alternativos em uma ampla variedade de ecossistemas (May 1977, Tilman 1982, Harrison 1986, Chase 1999). Estes estados estáveis alternativos são definidos para estabelecer uma co-existência sob as mesmas condições ambientais quando não existem perturbações e têm sido descritos através de modelos matemáticos ou empiricamente nos ecossistemas marinhos, lagos pouco profundos, bosques e oceanos (Scheffer *et al*. 1993, Scheffer *et al*. 2001, Chase 2003, Petraitis e Dudgeon, 2004). As mudanças graduais como, por exemplo, a

perturbação por pastagem, pode resultar num colapso repentino de um estado estável alternativo (Scheffer *et al* 2001). Estes câmbios podem gerar alterações na resistência frente a fatores ambientais e podem desencadear um pequeno distúrbio, provocando uma mudança ao atingir um limiar crítico. Um trabalho recente mostra que a recuperação dos ecossistemas a pequenas perturbações pode ser mais lento quando se está perto de uma transição descontínua, um fenômeno chamado desaceleração (Dakos *et al*. 2008). Por outra parte, o aumento da variabilidade e as mudanças de assimetria como biomassa e diversidade em períodos de tempo tem sido associado com mudanças rápidas nos ecossistemas (Carpenter e Brock 2006, Guttal e Jayaprakash 2009). No entanto, o inconveniente no uso destes parâmetros como indicadores é que exigido longos períodos de medição (Biggs *et al*. 2009, Scheffer *et al*. 2009). Portanto, mudanças nos padrões espaciais poderiam ser indicadores mais potentes quando um ecossistema está se acercando a uma mudança não continua (Rietkerk *et al*. 2004a, Scheffer *et al*. 2009, Dakos *et al*. 2010).

Descrição da área de estudo

Uma das áreas testadas é o Rio Tumbes com 1893,4 km^2, com elevações que variam de 885 msnm. Geograficamente, o Rio Tumbes fica entre os 03 º 30 'e 04 º 15' de latitude sul e 80 º 07 'e 80 ° 40' de longitude oeste. Mostrando características de precipitação máxima em fevereiro com valores 800-2500 mm / ano e menos precipitação durante o período de julho a outubro, com valores de 0,0 mm a 20 mm / ano.

O regime térmico registrou um comportamento variável em sua distribuição espacial e temporal, registrada em março as maiores temperaturas médias, que variam entre 28 º C

e 26 º C, e as temperaturas mais baixas, em agosto, a 23 º C e 26 º C. (INRENA, 1992; SENAMHI, 1976).

O ecossistema manguezal tem caracteristicas de precipitação anual é geralmente inferior a 100 mm. A média de temperatura varia entre 22 e 27 º C. Em geral, os solos têm um empate, com uma cor escura e são permeáveis. Eles são muito salgado reação forte, a reação extremamente ácida, alcalina excepcionalmente (INRENA. 2007).

O sistema bacteriano consiste em bactérias imersas numa camada de muco formando os biofilmes, os quais são abundantes tanto em água doce como em águas marinhas que cobrem a superfície de substratos submersos (Blum 1957; Rott, 1991; Patrick 1961, Stevenson e Pan, 1999). Estes biofilmes fototróficos são responsáveis por uma fração significativa da produção primária total (Underwood e Kromkamp 1999) e constituem a principal fonte de alimento para a macrofauna nos estuários (Herman *et al*. 2000) e dos ecossistemas de água doce (Stevenson, 1996). A estrutura destes biofilmes pode ser bastante irregular, tendo uma superfície ondulante, com uma distribuição em relação com a função das populações no biofilme, de acordo com observações feitas a diferentes escalas (Xavier *et al*. 2009). Por outro lado, o papel das interações biofísicas na formação da estrutura espacial a diferentes escalas espaciais está sendo cada vez mais estudado, sendo que as repercussões no funcionamento do ecossistema e os processos destas interações biológicas e físicas têm sido avaliadas. Sua distribuição ubíqua, taxonomia conhecida e a alta representatividade dos consórcios bentônicos tem feito dos biofilmes uma ferramenta valiosa, amplamente utilizada para avaliar as condições e mudanças característica do meio ambiente próprios dos sistemas aquáticos (Stoermer e Smol, 1999, Wassens *et al*., 2011, Chaves *et al*., 2012).

Dentro das diversas formas de interação, com frequência as bactérias se associam às superfícies biológicas. Esta associação pode resultar na formação de uma microflora própria como o primeiro passo de uma infecção ou uma interação benéfica. No meio aquático, as bactérias viajam com facilidade entre os habitats e os hospedeiros, sendo fundamental para as interações naturais entre hospedeiros e bactérias e para a produção de larvas de moluscos e ou sucesso de assentamento (Olafsen *et al*. 2003). Dentre os organismos que interatuam com bactérias estão os bivalves, emeles se acumulam grandes quantidades de micro-organismos da água de mar que ingressam por filtração sendo o gênero *Vibrio* o predominante. Este gênero cumpre um papel dinâmico em os ambientes de agua marnha e salobro. Este gênero está associado geralmente com as superfícies de invertebrados e também na hemolinfa e órgãos internos de espécies de bivalves saudáveis (Olafsen *et al*, 1993, Riquelme 2003).

A maioria dos animais e as plantas obtém simbiontes microbianos essenciais de transferência horizontal, o processo pelo qual uma série de organismos é colonizada por micro-organismos específicos adquiridos do meio ambiente circundante depois da embriogênese. Devido a que estes simbiontes potenciais representam só uma pequena fração do consórcio microbiano do ambiente, as espécies animais devem desenvolver mecanismos pelos quais aumentam a probabilidade de serem colonizadas pelos micróbios apropriados, desalentando ao mesmo tempo a colonização por outros inespecíficos. Estas características sugerem que o estabelecimento de uma simbiose em um hábitat aquático representa um desafio e, até agora, os mecanismos de evolução dos animais aquáticos para superar essas limitações e garantir a colonização de simbiontes não tem sido muito estudado ainda.

Deve notar-se que a bactéria parede constituição confere propriedades aniónicas, oferecendo elevada afinidade e atracção para os metais, como o cobre catiónicos (Ferris, 1990). As paredes celulares de bactérias são aniónicos notável na sua capacidade para fixar os metais e proporcionar sítios para a nucleação e crescimento dos minerais. Beveridge e Fyte (1985) mostraram tipos de polímeros da parede celular, que são responsáveis pela ligação de metal em paredes de bactérias Gram-positivas e Gram-negativas.

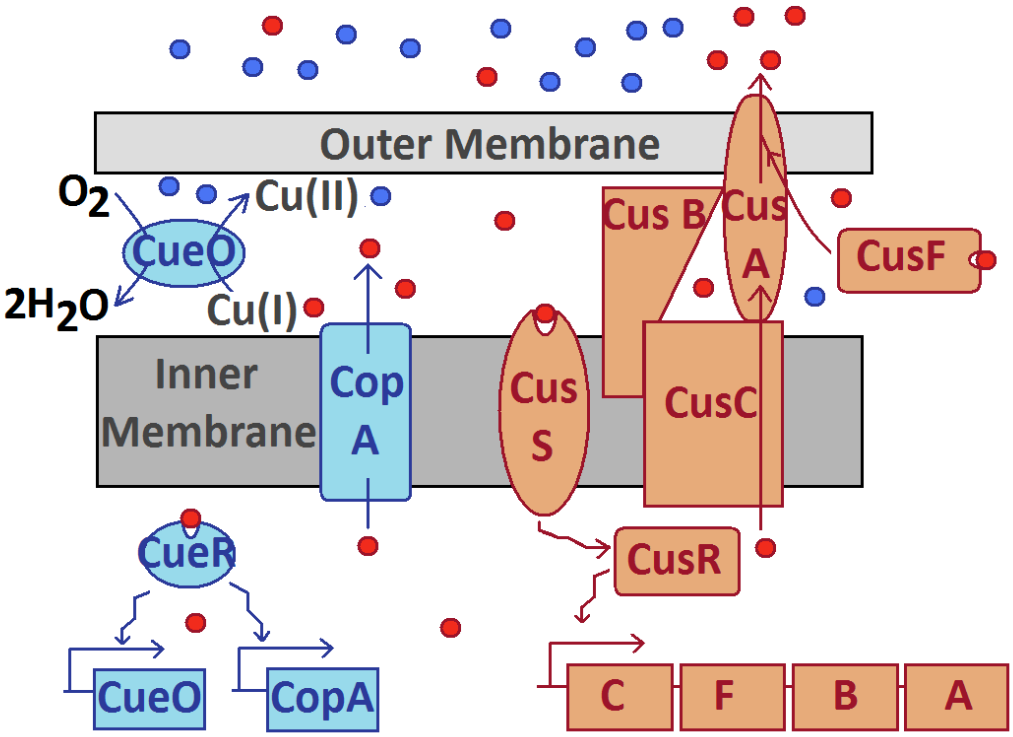
Algumas comunidades de bactérias estão dominadas por uma ou poucas espécies, enquanto que outras constam de numerosas espécies que convivem juntas. São várias as explicações propostas para explicar a diversidade aparente em comunidades naturais. Em referência aos ecossistemas em geral, Connell (1978) postula que a variabilidade temporal e espacial no entorno freia a progressão da exclusão competitiva e permite uma maior riqueza específica. Posteriormente, este conceito foi formalizado como a hipótese de distúrbio intermédidiario. Apôs seus estudos sob o fitoplâncton nos Grandes Lagos, Tilman (1982) propus a hipótese da relação dos recursos, o que explica a co-existência de espécies de algas de uma fonte equilibrada para que as diferentes espécies que estão limitadas pelos distintos recursos, outorguem estabilidade ás múltiplo espécies do consórcio. Estes fatores em conjunto também podem determinar a composição de espécies de microorganismos bentônicos. Um maior número de espécies bentônicas pode estar relacionado com uma maior heterogeneidade espacial associada à vida bentônica. Portanto, é importante a identificação dos vários fatores que potencialmente podem regular a composição destes consórcios bacterianos.

O descrito acima estabelece basicamente que os fatores que controlam as bactérias bentônicas podem se classificar nos processos de regulação da acumulação de biomassa específicos de cada área e as que regulam os processos que ocorrem para controlar a perda de biomassa. O principal fator que regula a acumulação de biomassa é a taxa de crescimento numa condição de recursos limitados, modulada por fatores tais como a contaminação, temperatura, nutrientes, pH e metais como o cobre. O fato da população humana estar em expansão e o aumento associado das atividades industriais e agrícolas são alguns dos fatores de pressão nos ecossistemas aquáticos. As atividades antropogênicas tem mudado o clima global e dos hábitats, aumentando o aporte de alguns nutrientes e de um grande número de substâncias químicas, sendo que a contaminação da água tem afetado muito a distribuição das espécies aquáticas. Fatores de estresse antropogênico provavelmente alterem a abundância de espécies e a persistência, o qual reduz a biodiversidade (Mooney e Godron 1983).

Cobre em bactérias e algas

O cobre é um micronutriente essencial, que serve como co-factor para numerosas enzimas. No entanto, o cobre em excesso também é citotóxica. Concentração em que cobre inibe o crescimento de *E. coli* têm sido relatados para variar de sub níveis micromolares em estudos utilizando meios de crescimento definidos contendo uma fonte de carbono simples (Macomber e Imlay, 2009). para os níveis milimolares em estudos utilizando meios complexos. O cobre pode existir como Cu (I) e Cu (II) e o ciclo redox entre estes dois oxidação estados pode gerar prejudiciais espécies reativas de oxigênio em células aeróbias (Buettner e Jurkiewicz, 1996; Macomber, *et al*., 2007). Cobre toxicidade também é atribuída à sua capacidade para interferir com outros cofactores metálicos de enzimas ou causar misfolding proteína ou agregação, levando à

toxicidade do cobre, na ausência de oxigênio (Macomber e Imlay, 2009). Em *E. coli*, dois sistemas cromossomicamente codificadas, cus e Cue, são induzidas sob estresse cobre e estes dois sistemas de código para diversas proteínas que têm a função de proteger a bactéria contra o estresse de cobre (Outten *et al*., 2001; Rensing e Grass, 2003). Os cus códigos do sistema para a bomba CFBA Cus que é principalmente responsável pela translocação de cobre citoplasmático e periplasmático para fora da célula bacteriana sob condições anaeróbicas, (Grass e Rensing, 2001; Loftin *et al*., 2005). Os códigos do sistema de sinalização para a Copa e CueO. COPA é Cu (I) de translocação do tipo p-ATPase de que as bombas de Cu citoplasmáticas (I) para o espaço periplásmico da bactéria e CueO é uma oxidase multicopper (MCO), uma família de enzimas que contêm quatro átomos de cobre e dois a oxidação de vários substratos para a redução de electrões 4 de dioxigénio a água Que mecanismos pelos quais a bactéria Escherichia coli sentidos e responde a ligandos extracelulares. Um exemplo do primeiro mecanismo é a sua resposta ao excesso de cobre, o que induz a expressão de proteínas reguladoras de cobre funcionem para proteger a bactéria contra a toxicidade induzida por cobre. Um exemplo de outro mecanismo é a sua resposta a quantidades vestigiais de certos produtos químicos que podem ser útil ou prejudicial para a bactéria e a bactéria responde movendo para perto ou para longe deles, respectivamente. A maior parte da informação são abouth a estrutura e actividade da enzima CueO multicopper oxidase e como ela se relaciona com o mecanismo da tolerância ao cobre transmitida por CueO (Grass e Rensing, 2001).



Homeostase de cobre em *E. coli*. Os pequenos círculos representam os íons de cobre (vermelho para Cu (I) e azul para Cu (II)). Os genes de C, F, B, A. Codificar o CUSC, CusF, CusB e proteínas CUSC, respectivamente CusF é uma acompanhante de cobre periplasmático enquanto Cusa, CusB e CUSC juntos formam uma bomba de efluxo de cobre abrangendo ambas as membranas. Cuss CusR e formam um par sensor / regulador, que induzem a expressão dos genes cusCFBA na presença de Cu (I). Cuer é um activador transcricional que induz a expressão de proteínas e a COPA CueO sobre Cu (I) de ligação. CueO é uma oxidase que oxida multicopper Cu (I) e a COPA é do tipo P-ATPase que as bombas de Cu citoplasmática (I) para o espaço periplasmático (Satish, 2009).

A estrutura de consórcios bacterianos nos rios podem ser afetados por níveis elevados de produtos químicos, além dos recursos naturais. Os metais são ainda uma das fontes mais comum de contaminação ambiental e, da mesma forma que a contaminação orgânica, origina-se tanto da deposição atmosférica difusa, assim como

de várias fontes pontuais como as minas e fundições (EEA 1994, EEA 2012). A sensibilidade aos metais por parte das bactérias de água doce e as comunidades de diatomáceas tem sido avaliada em condições de campo e de laboratório (Say e Whitton 1981, Leland e Carter 1984; Deniseger *et al.* 1986, Genter *et al* 1987, Gustavson e Wangberg 1995, Gray e Hill 1995, Genter 1996, Medley e Clements 1998, Paulsson *et al.* 2000, Munees e Abdul 2012, Torres *et al*. 2012), mas o número de estudos efetuados até agora é limitado. A sensibilidade específica aos metais pode se refletir também nas mudanças na abundância relativa das espécies nas comunidades. Uma exposição prolongada a uma substancia tóxica pode causar uma subtituição de espécies de bactérias e algas sensíveis por outras mais tolerantes e, finalmente, induzir a adaptação das espécies individuais (Blanck *et al.* 1988). Desta forma, de acordo com Blanck *et al.* (1988), a tolerância da comunidade em seu conjunto irá se incrementar, processo denominado tolerância induzida na comunidade pela poluição ou PICT. Este conceito tem sido validado em comunidades de estuários submetidos a vários compostos químicos (Blanck e Wangberg 1988a, b, Blanck e Dahl 1996, Demolin e Bååth 2008, Fechner *et al*. 2011) através da medição de respostas fisiológicas em algas e comunidades bacterianas. A abordagem PICT também tem sido aplicada recentemente às comunidades de perifitôn de água doce submetidas a estresse por metais (Gustavson e Wangberg 1995, Paulsson *et al.* 2000, Soldo e Behra 2000, Fechner *et al*. 2011). Experimentos independentes em monocultivos de bactérias mostram grandes diferenças na tolerância aos metais entre as cepas de bactérias da mesma espécie provindo de ambientes contaminados e não contaminados (Jensen *et al.* 1974, Say e Whitton 1977, Foster 1982, Takamura *et al.* 1989, Sundar *et al.* 2010).

A presente Tese centrou-se nas comunidades bacterianas presentes nos sedimentos, avaliando as respostas apresentadas pela comunidade bacteriana frente a diferentes agentes estressores que podem influenciar na composição das espécies dos consórcios bacterianos. Os estudos realizados, por um lado, incluíram as respostas antioxidantes das espécies individuais isoladas de fontes naturais em relação ao processo de sucessão em um cenário estressado pelo cobre. Por outro lado foi avaliada a dinâmica destas comunidades frente a diversos fatores ambientais, como temperatura, nutrientes, e pH. Finalmente foi estudada a resposta de recuperação das comunidades no que diz a respeito à influência dos fatores citados acima, no intuito de gerar informação das características que fazem destes biofilmes comunidades efetivas em ambientes particulares.

No contexto teórico apresentado, é importante desenvolver técnicas que permitam avaliar o risco de metais como o cobre nos ecossistemas. Além disto, poucos estudos têm sido realizados em ecossistemas moderadamente contaminados por metais. No entanto, a discriminação entre os efeitos dos metais e o impacto das condições naturais e as propriedades dinâmicas das comunidades microbianas é ainda uma pergunta a ser respondida e, por isto, formou parte dos objetivos levantadas por esta Tese.

2. OBJETIVOS

2.1. Objetivo geral

Analisar a composição de spp. e atividade antioxidante das comunidades bacterianas associadas do biofilme durante seu desenvolvimento em ambientes aquáticos com diferentes graus de estresse por cobre.

Associados a este Objetivo Geral foram levantados os seguintes objetivos específicos:

1- Verificar experimentalmente o impacto de cobre nos consórcios de sucessão semi-natural bacteriano.

2- Relacionar a diversidade microbiana e a contaminação de cobre com a capacidade antioxidante de consórcios bacterianos associados do biofilme.

3- Avaliar o efeito gerado pelo diversos fatores ambientais e cobre na alteração da diversidade bacteriana associadas do biofilme.

4- Verificar o potencial de indução na tolerância pela poluição e recuperação dos biofilmes expostos a cobre.

- CAPÍTULO 1 -

Influence of copper on biofilm bacterial communities and possible implication in succession dynamics

Rafaela Elias Letts[1,2]*, Carla Mere[2], Paola Yurivilca[2], Silvia Sato[2], José M. Monserrat[1,3]

Manuscrip send to Microbial Ecology

[1]Programa de Pós-Graduação em Ciências Fisiológicas - Fisiologia Animal Comparada, Universidade Federal de Rio Grande – FURG, Rio Grande, Brazil.

[2] Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Lima, Perú

[3]Instituto de Ciências Biológicas (ICB), Universidade Federal do Rio Grande – FURG, Rio Grande, Brazil

* Corresponding author

Av. Honório Delgado 430, San Martín de Porres. Lima-Peru

rafaela.elias@upch.pe, releltts@hotmail.com

Resumo

A dinâmica da composição de espécies em biofilmes dos ecossistemas naturais é conhecido por ser regido por fatores físico-químicos, a interação entre espécies e de perturbação das comunidades por diversas variáveis estressantes como a poluição. Avaliaram-se estes dinâmica de um sistema de modelo de sucessão, a fim de analisar os efeitos da poluição de metal de cobre em mudanças na diversidade de bactérias e a sua capacidade antioxidante total como um parâmetro de sub-letal para medir a tensão em cada uma das espécies isoladas durante o desenvolvimento do biofilme. Os resultados mostraram uma redução importante na diversidade após o tratamento de cobre. Foi estudada local RT (de água doce e de alto impacto com metais rio Tumbes, em Tumbes (Peru)) e P (estuarinos água e metais de baixo impacto, um canal estuarino Tumbes, sendo a diversidade de bactérias mais elevada do que na RT em P). A prevalência de *Pseudomonas sp*. no local P sugerem que este género é importante para a fase inicial do desenvolvimento do biofilme e no local RT *Aeromonas trota* mostrou ser um importante indicador de locais contaminados. As alterações observadas na diversidade de bactérias e a sua capacidade antioxidante deve ajudar na compreensão dos mecanismos provocada por organismos aquáticos de aumentar a sua tolerância nesses ecossistemas. Em conclusão, este estudo mostrou que os biofilmes de um metal ambiente de baixo impacto foram mais severamente afetados pela exposição ao cobre durante uma fase inicial de desenvolvimento do que durante a maturidade, em contraste com biofilmes de alto impacto ambiental. Este impacto pode ser regulada por estresse oxidativo modulação, como mostrado pelos resultados da capacidade antioxidante total do género *Pseudomonas* e *Bacillus*, que mostrou diferenças significativas após a exposição ao cobre, quando comparada com o grupo de controlo.

Palavras-chave: ecossistemas de mangue, biofilme, metais pesados, radiação, capacidade antioxidante de cobre.

Abstract

The dynamics of species composition in natural ecosystem biofilms is known to be governed by physico-chemical factors, interaction between species and disturbance of communities by several stressful variables including pollution. It was evaluated these dynamics with a succession model system in order to analyze the effects of copper metal pollution on changes in bacterial diversity and their total antioxidant capacity as a sub-lethal parameter to measure stress in each isolated species during biofilm development. The results showed an important decrease in diversity after copper treatment. It was studied site RT (fresh water and high-impacted with metals, Tumbes river in Tumbes (Perú)) and P (estuarine water and metal low-impacted, an estuarine channel in Tumbes, being the bacteria diversity higher in RT than in P). The prevalence of *Pseudomona sp*. in P site suggest that this genus is important for the initial phase of biofilm development and in RT site *Aeromonas trota* showed to be an important indicator of polluted sites. The observed changes in bacterial diversity and their antioxidant capacity should aid in the understanding of mechanisms elicited by aquatic organisms to enhance their tolerance in these ecosystems. In conclusion, this study showed that biofilms from a metal low-impacted environment were more severely affected by copper exposure during an early developmental stage than during maturity in contrast with biofilms from high-impacted environment. This impact may be regulated by modulating oxidative stress as shown by the results of total antioxidant capacity for the genera *Pseudomonas* and *Bacillus* that showed significant differences after copper exposure when compared with the control group.

Key words: mangrove ecosystems, biofilm, heavy metals, radiation, antioxidant capacity, copper

1. Introduction

The diverse aquatic habitats on the earth include mangrove ecosystems, where bacterial populations has important roles in food webs and nutrient cycles. Also, their high surface to volume ratio, their intimate contact and interaction with the environment suggest that microorganisms should be very sensitive and responsive to environmental stress (Brookes, 1995; Giller *et al*., 1998; Boor, 2006; Amir *et al*., 2009). Microbenthic bacterial consortia are organized in multilayered structures that cover the illuminated submersed substrates (Blum, 1957; Boles *et al*., 2004). These structures are complex associations of bacterial, algae, detritus and inorganic particles, immerse in polymeric secretions, forming an intricate network of functional interactions (Wetzel, 1993; Sobczack, 1996) known as biofilms. Correlations between aquatic physico-chemical conditions and the occurrence of diatoms and bacterial species in the field have been used to interpret the preference of taxa to single selected environmental factors and, in fact, the distribution of diatoms taxa has been used to infer certain environmental characteristics of aquatic systems (Anderson *et al*., 1993; Van Dam *et al*., 1994; Stevenson and Pan, 1999; Boor 2006; Foster *et al*., 2011). Also, radiation influences directly marine and continental ecosystems (Shiu & Lee, 2005), where a decline in primary productivity by UV radiation, affects other throphic levels of the chain (Hader *et al*., 2007; Rastogi *et al*., 2010). Within the three types of UV radiation (UVA: 315-400 nm; UVB: 280-315 nm; UV-C: <280 nm), UV-B produces adverse effects in different habitats, causing damages in several organisms including bacteria species (Rastogi *et al*., 2010), generating the organic compound production of low molecular weight CPDs (cyclobutane pyrimidine dimers) and Reactive Oxygen Species (ROS) (Hader *et al*., 2007). To cope and neutralize ROS, exists a system of defences conformed by enzymatic and non-enzymatic antioxidants. The imbalance between pro-

oxidants and antioxidants lead to an oxidative stress condition (Halliwell and Gutteridge, 2007). It is known that ROS destabilize oxidized cellular membranes (Shiu & Lee, 2005) and inhibit the photosynthetic activity of bacteria and phytoplankton (Hader *et al*., 2007).

Between different stressful factors that face the aquatic organisms, like UV radiation and metals, the microorganisms cope with the produced ROS through the antioxidant defence system. As mentioned above, this mechanism of defence displays enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and non-enzymatic molecules like carotenoids (CAR), ascorbate (ASA), α-tocopherol (α-TOC) and reduced glutathione (GSH) (Srivastava, 2010).

Metals are still one of the most common sources of environmental contamination, and several authors reported susceptibility and diversity changes of freshwater bacterial communities (Barranguet *et al*., 2003; Boivin *et al*., 2005; García-Meza *et al*., 2005; Serra *et al*., 2010). Bacteria species-specific sensitivities to metals may be also reflected in changes in the relative abundance and diversity at the community level. A prolonged exposure to stressful factors may induce a replacement of sensitive species by more tolerant ones and ultimately may induce adaptation on individual species (Boivin *et al*., 2005; Boivin *et al*., 2006). Some essential (i.e.: copper and iron) and non-essential (cadmium and arsenic) elements share some toxic mechanisms that include oxidative stress generation (Lu *et al*., 1999; Ventura-Lima *et al.*, 2011).

The aim of the present study was to evaluate the effect of copper exposure in bacterial diversity together with the total antioxidant capacity of bacteria associated to biofilm during a succession event for two communities origined from two sites with different levels of metal pollution.

2. Materials and methods

2.1. Test site and sampling

The mangroves studied are located at the mouth of the Tumbes River, in Peru (Fig. 1). It was established two sites to perform the studies: (1) Tumbes River (RT), a region extremely and chronically polluted by a former polymetalic mine and with characteristic of fresh water; and (2) Faculty station (P), a low pollution site presenting a mixture of saline and fresh estuarine water.

2.2. Analysis of water of mangrove and mud

The characteristic of water and mud was determined two times by year, in the humid and dry seasons (February and July, respectively). The parameters evaluated were dissolved oxygen concentration, pH, temperature, conductivity and particles in suspension, being determined *in situ* in all site studies with a multi-parametric equipment (WTW). Nitrate, phosphates and potassium were measured using a colorimetric kit (HANNA, model HI 83225 Grow Master); UVB intensity was determined by a Luxometer (Delta OHM). Metal concentration in water and mud was analyzed by Optic-ICP.

2.3. Succession development

This measurement was made for dry and humid season. Glass slides (7.5 x 2.5 cm^2 surface) were used as an artificial and selective substrate for bacterial attachment. Before use, glass slides were soaked in a 10% HNO_3 (grade reagent) solution for 24 h and then rinsed with deionizated water. The slides were placed inside polyethylene tanks with 50 L of re-circulated water taken from the two studied sites, each tank contained 70 slides. In parallel, in another system, it was reproduced the succession development using water from RT or P containing 20 µmol/L of nominal copper (as $CuCl_2$), a sub-lethal concentration for bacteria species (Boivin *et al*., 2006). Assays were performed in triplicate.

After the first week period, 10 glass slides were removed from each tank and it was taken a sample of the early biofilm formed for bacterial isolation in TSA Tryptone Soya Broth agar (Merck) and incubated at 25 °C. This procedure lasted 4 weeks until the development of a climax biofilm. Each colony forming units (CFUs) was removed and put to grow in Nutritive Medium (Merck) with constant shaking at 25°C until reaching an optical density (OD) of 0.6.

2.4. Measurements of microbenthic activity

Total antioxidant capacity was measured with culture at 0.6 OD with a methodology adapted from Rice-Evans *et al*. (1994), using 2,2-azinobis(3-ethylbenzothiazoline 6-sulfonate) reagent (DPPH). Each sample from control and copper exposed groups were lised with a sonicator for 15 min at 60 Hz (BRASONIC ultrasonic cleaner, model 3510) and measured in triplicate in 96 well plates, being registered the absorbance at 517 nm

using an ELISA reader (VERSA max microplate reader). The total antioxidant capacity was estimated according to the following formulae:

$CA\% = [1- (A_2-A_3)/A_1] \times 100$,

were: A_1: Absorbance of standard of reference DPPH solution, A_2: sample absorbance and A_3: blank absorbance

Viability assays were carried out in 96 well plates following a modified methodology described by Palomino *et al*. (2002). After the incubation with DPPH, 100 µL of the suspension was used as an inoculum, 30 µL of resazurin solution 0.01% (w/v) was added to each well, incubated overnight at 37 ºC and assessed for color development at 570 nm in a microplate reader (Bio-Tek Instruments). The viability percentage was calculated considering 100% for the samples of the control group.

2.5. Molecular analysis

DNA of each colony was extracted from cell cultures using the Wizard® Genomic DNA Purification kit (PROMEGA Corp., Madison, WI, USA), following suppliers instructions. Extraction products were visualized on 1% agarose gel with GelRed® (Biotium).

Primers of 16S segment were designed as described previously (Ritchie, 2008). DNA samples were amplified through polymerase chain reaction (PCR), performed according to Ritchie (2008) except for primer annealing temperature, optimized for 58 °C. PCR products were analyzed on GelRed®-stained 1% agarose gel using Low DNA Mass Ladder (Invitrogen) was employed as molecular weight marker and then purified using the enzymes exonuclease I (Exo) and shrimp alkaline phosphatase (SAP). Purified PCR products were sequenced using MegaBACE 1000 automated sequencer. Resulting

chromatograms were analyzed and DNA sequences were blasted using NCBI-BLAST searches of GenBank®.

2.6. Bray-Curtis similarity index

The Bray-Curtis community similarity index (Bray and Curtis 1957; Clarke and Warwick, 1994) was calculated every week between samples of succession development for RT and their copper treatment for dry and humid season and the same procedure was followed for P samples, using the formulae:

$$S'_{jk} = 100 * \left(1 - \frac{\sum_{i=1}^{n} \left| y_{ij} - y_{ik} \right|}{\sum_{i=1}^{n} \left(y_{ij} + y_{ik} \right)} \right)$$

Being n: number of taxa, *j*: community j, *k*: community k, y_{ij}: fraction of taxa *i* in community *j*, y_{ik}: fraction of taxa *i* in community *k*, S'_{jk}: similarity value between communities *j* and *k*.

2.7. Statistics

Physico-chemical parameters were evaluated by Principal Component Analysis (PCA). Significant differences in total antioxidant capacity between the weeks of development of biofilm and for each different season were analysed using two-way ANOVA, factors being metal exposure and season (Sokal and Rohlf, 1981). Differences in the abundance of species between the communities RT and P were also tested with one-way ANOVA.

Assumptions of ANOVA (normality and variance homogeneity) were previously verified and mathematical transformations applied if at least one was violated. Post-hoc comparisons of means were performed using Bonferroni test. In all cases type I error was fixed in 0.05.

3. Results

3.1 Physico-chemical characteristics of the sites

Cu, Zn, As, Pb and Cd concentrations were one to two orders of magnitude higher at RT than at P (Table 1). Differences in water conductivity should be related to the presence of fresh water at RT and saline/estuarine water at P (Table 1). The pH in all streams was neutral and values were similar between sites.

Table 1. Mean and SD values corresponding to physical and chemical parameters of water determined during the experimental period (2009-2011). Limit ISQG (Iterim Sediment Quality Guideline) for copper = 35.7 mg/kg and Limit PEL (Permissible Exposure Limit) for copper = 35 mg/kg. All the measure was by triplicate.

	Tumbes river (RT)				Fishing station (P)			
Parameter	Humid		Dry		Humid		Dry	
	mean	S.D.	Mean	S.D.	mean	S.D.	mean	S.D.
pH	7.4	0.1	7.88	0.1	7.4	0.3	7.6	0.0
Dissolved oxygen (%)	18.2	3.3	20.3	1.2	13.0	1.2	17.0	0.2
Particules suspention (ppm)	250.0	10.8	180.0	43.1	2,000.0	12.6	2,000.0	10.8
Temperature (°C)	26.2	0.2	25.4	1.1	27.6	0.9	26.0	1.7
Conductivity (µS)	127.0	2.3	105.0	55.6	3,999.0	0.0	3,999.0	0.0

UVB (W/m^2)	435.9	91.2	370.0	24.1	17.5	1.4	16.0	2.1
Potassium (mg/L) in water	1.0	0.1	1.0	0.2	0.0	0.0	0.0	0.0
Cu (mg/Kg) in sediment	986.4	65.3	1,790.4	108.3	214.4	11.6	214.4	28.3
Zn (mg/Kg) in sediment	1,286.45	102.0	1,086.5	66.3	157.9	18.4	157.5	15.0
As (mg/Kg) in sediment	577.0	36.1	557.5	29.6	7.7	0.4	7.75	3.3
Pb (mg/Kg) in sediment	667.0	24.6	656.0	35.5	28.5	2.1	23.5	8.3

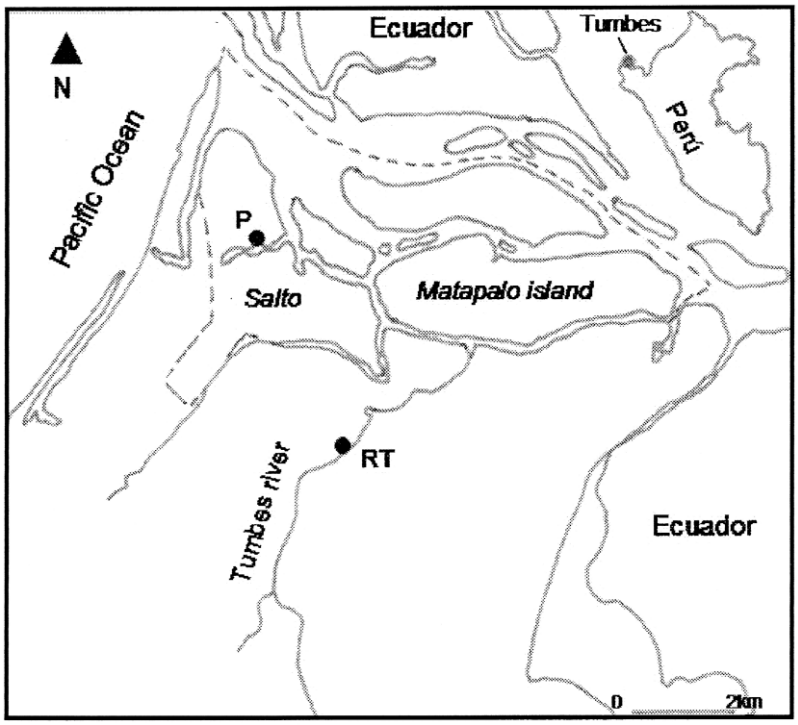


Figure 1. Location of the experimental sites in Tumbes mangrove: RT (Tumbes river; 17560527E 9605246N UTM) and P (Faculty station model 17567257E - 9612703N UTM).

A.

	Treatment without Cu					Treatment with Cu			
Week	**1**	**2**	**3**	**4**	***Week***	**1**	**2**	**3**	**4**
Pseudomonas fluorescens	88	0	14	0	*Pseudomonas fluorescens*	96	0	0	0
Pseudomonas sp.	1	0	0	0	*Pseudomonas syringae*	4	0	0	0
Shewanella algae	3	0	0	0	*Uncul Bacillus sp. clone DH15_47*	0	0	0	0
Bacillus sp.	0	17	24	31	*Uncul bacterium clone (ncd1072f03c1)*	0	0	0	0
Bacillus pumilus	0	56	0	0	*Uncul bacterium isolate (PGBw25)*	0	95	0	0
Bacillus altitudinis	0	28	0	0	*Aeromonas punctata*	0	4	0	0
Bacillus thuringiensis	0	0	33	0	*Uncul beta proteobacterium clone S31*	0	0	2	0
Uncul Bacillus sp.	0	0	7	0	*Pseudochrobactrum saccharolyticum*	0	0	16	0
Uncul bacterium gene, clone: NG_inoculum_12	0	0	21	0	*Uncul bacterium (UTFS-R119-101)*	0	0	82	0
Enterobacter sp.	0	0	0	12	*Delftia sp.*	0	0	0	15
Aeromonas trota	0	0	0	43	*Lysinibacillus sphaericus*	0	0	0	32
Clostridium bifermentans	0	0	0	14	*Pseudomonas putida*	0	0	0	28
					Myroides sp.	0	0	0	1
					Pseudomonas sp	0	0	0	24

B.

	Treatment without Cu					Treatment with Cu			
Week	**1**	**2**	**3**	**4**	***Week***	**1**	**2**	**3**	**4**
Pseudomonas fluorescens	100	38	33	0	*Shewanella algae*	1	0	0	0
Bacillus sp.	0	12	0	31	*Pseudomonas fluorescens*	99	0	0	0
Uncul bacterium clone (ncd2256g11c1)	0	12	0	0	*Pseudomonas syringae*	1	0	0	0
Exiguobacterium sp.	0	19	0	0	*Brevibacillus brevis*	0	0	0	0
Bacillus altitudinis	0	19	0	0	*Uncul Bacillus sp. clone DH15_47*	0	0	0	0
Lysinibacillus fusiformis	0	0	3	0	*Uncul bacterium clone (ncd1072f03c1)*	0	0	0	0
Bacillus thuringiensis	0	0	27	0	*Uncul bacterium isolate (PGBw25)*	0	95	0	0
Uncul bacterium gene	0	0	6	0	*Aeromonas punctata*	0	4	0	0
Uncul bacterium gene, clone: NG_inoculum_12	0	0	18	0	*Alpha proteobacterium*	0	0	2	0
Bacillus pumilus	0	0	12	0	*Uncul beta proteobacterium clone S31*	0	0	5	0
Enterobacter sp.	0	0	0	12	*Uncul compost bacterium, clone PS165*	0	0	15	0
Aeromonas trota	0	0	0	43	*Pseudochrobactrum saccharolyticum*	0	0	76	0
Bacillus sp	0	0	0	0	*Uncul bacterium clone (UTFS-R119-101*	0	0	2	0
Clostridium bifermentans	0	0	0	14	*Aeromonas trota*	0	0	2	0
					Delftia sp.	0	0	0	15
					Lysinibacillus sphaericus	0	0	0	32
					Pseudomonas putida	0	0	0	28
					Myroides sp.	0	0	0	1
					Pseudomonas sp	0	0	0	24

Figure 2. Relative abundance of species (based in the number of colonies forming units, CFUs) in the plate agar culture during development of bacterial consortia with and without copper exposure during humid season (A) and dry season (B) from RT site.

A.

	Treatment without Cu					Treatment with Cu			
Week	**1**	**2**	**3**	**4**	***Week***	**1**	**2**	**3**	**4**
Pseudomonas sp.	4	0	0	67	*Exiguobacterium sp.*	0	5	0	0
Pseudomonas putida	19	0	0	0	*Uncul pseudomonas sp*	0	2	0	0
Pseudochrobactrum saccharolyticum	1	2	0	0	*Bacillus thuringiensis*	0	2	0	0
Uncul prokaryote isolate DGGE C-16S-250707	77	0	0	0	*Bacillus cereus*	0	18	0	0
Bacillus altitudinis	0	14	0	4	*Bacillus sp.*	0	73	50	8
Uncul bacterium clone (ncd2595h01c1)	0	4	0	0	*Pseudochrobactrum saccharolyticum*	0	0	6	0
Bacillus pumilus	0	80	0	12	*Pseudomonas putida*	0	0	6	0
Bacillus subtilis	0	0	17	0	*Proteus myxofaciens*	0	0	11	0
Uncul bacterium gen, isolate: S01_D08	0	0	33	0	*Pseudomonas sp*	0	0	28	0
Rumen bacterium	0	0	17	0	*Bacillus subtilis*	0	0	0	3
Bacillus sp	0	0	17	8	*Bacillus altitudinis*	0	0	0	10
Delftia sp.	0	0	17	0	*Uncul bacterium clone (aaa52h05)*	0	0	0	17
Lysinibacillus sphaericus	0	0	0	4	*Lysinibacillus sp*	0	0	0	2
Myroides sp	0	0	0	4	*Uncul bacterium clone DF6022*	0	0	0	59

B.

	Treatment without Cu					Treatment with Cu			
Week	**1**	**2**	**3**	**4**	***Week***	**1**	**2**	**3**	**4**
Pseudomonas sp.	4	0	0	64	*Pseudomonas sp.*	2	0	28	0
Pseudomonas fluorescens	3	0	0	0	*Pseudomonas fluorescens*	24	0	0	0
Pseudomonas putida	18	0	0	0	*Pseudomonas putida*	73	0	6	0
Pseudochrobactrum saccharolyticum	1	2	0	0	*Uncultured pseudomonas sp*	0	2	0	0
Uncul prokaryote isolate DGGE C-16S-250707	75	0	0	0	*Bacillus thuringiensis*	0	2	0	0
Uncul bacterium clone	0	2	0	0	*Bacillus cereus*	0	18	0	0
Uncul bacterium clone (ncd2595h01c1)	0	4	0	0	*Exiguobacterium sp.*	0	2	0	0
Bacillus altitudinis	0	2	0	4	*Bacillus pumilus*	0	2	0	0
Bacillus pumilus	0	12	7	2	*Bacillus sp.*	0	64	50	8
Roseateles sp.	0	79	7	0	*Sporosarcina sp.*	0	9	0	0
Lysinibacillus sphaericus	0	0	7	12	*Pseudochrobactrum saccharolyticum*	0	0	6	0
Bacillus subtilis	0	0	7	0	*Proteus myxofaciens*	0	0	11	0
Uncul bacterium gen, isolate: S01_D08	0	0	13	0	*Bacillus subtilis*	0	0	0	3
Rumen bacterium	0	0	7	0	*Lysinibacillus sphaericus*	0	0	0	3
Bacillus sp	0	0	35	14	*Bacillus altitudinis*	0	0	0	10
Delftia sp.	0	0	7	0	*Uncul bacterium clone (aaa52h05)*	0	0	0	16
Lysinibacillus fusiformis	0	0	13	0	*Lysinibacillus sp*	0	0	0	2
Myroides sp	0	0	0	4	*Uncul bacterium clone DF6022*	0	0	0	57

Figure 3. Relative abundance of species (based in the number of colonies forming units, CFUs) in the plate agar culture during development of bacterial consortia with and without copper exposure during humid season (A) and dry season (B) from P site.

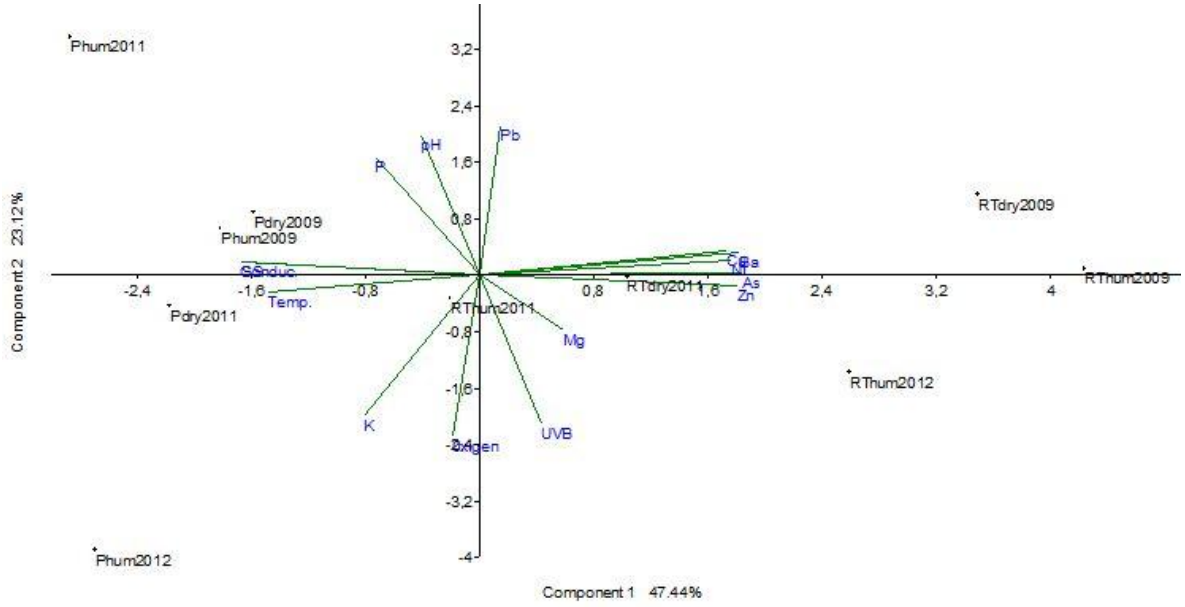


Figure 4. Principal component analysis (ACP). P or : RT : the sampling sites. Dry or Hum: the season (dry or humid) analysed. The year of take up data was also included.

Microphytobenthic communities associated with biofilm were diverse for every week: a total of 39 taxa were identified in this study. In general, bacteria were mainly represented by members of the motile *Pseudomonas* and *Bacillus* genera. Despite the general similarity between all samples, the copper exposure in this study revealed small shifts in species composition.

Differences in the bacteria communities from RT and P sites in humid and dry seasons and after copper treatment is shown in Figure 2 and 3 respectively. Even when communities changes were dependent on the origin (sampled site), communities from P site at the humid season were more similar to communities from the same site at the dry season, than communities from the P site exposed to copper at both seasons. A similar result was observed for communities from the RT site (Figure 2). Bray-Curtis similarity index indicated metal exposure differences (0.15 similarity) but not between seasons (0.8 similarity) within bacterial communities from both sites.

The result of total antioxidant capacity showed that some genera can regulate their antioxidant response after copper exposure (see Table 2). These genera did not showed a change of antioxidant capacity response at the different seasons ($p>0.05$). The results showed in the first week a modulation of total antioxidant capacity in *Pseudomonas fluorescens* RT station, this modulation is not present in the station P. From the second week the total antioxidant capacity is present and represented by *Bacillus sp*. showing increased in response antioxidant in copper treatments for dry season.

The PCA analysis showed that component 1 (47.44% of total variation) described the distribution of RT and P sites, been associated to metal composition. The second component accounted for 23.12% of total variation, being associated to pH and dissolved oxygen (Figure 4).

Table 2. Significative statistics differences ($p<0.05$) in total antioxidant capacity between treatment with copper and without copper for RT and P sites. Indicated genus or species are the more frequent in each experimental condition. The underline in blank space indicated no significant differences.

Site	Season	Treatment	Genus/Species	Week 1	*P*	Week 2	*P*	Week 3	*P*	Week 4	*P*
RT	Humid	with Cu	*Pseudomonas fluorescens*	83.9 (8.3)	0.0291	----	----	----	----	----	----
		Control	*Pseudomonas fluorescens*	47.7 (4.2)		----	----	----	----		
		with Cu	*Myroides sp*	----	----	----	----	----	----	24.1 (2.3)	0.004
		Control	*Myroides sp*	----	----	----	----	----	----	44.5 (4.2)	
	Dry	with Cu	*Pseudomonas fluorescens*	42.9 (0.8)	0.0341	----	----	----	----	----	----
		Control	*Pseudomonas fluorescens*	53.6 (3.2)		----	----	----	----	----	----
		with Cu	*Pseudomonas sp.*	----	----	----	----	41.4 (1.4)	0.0044	----	----
		Control	*Pseudomonas sp*	----	----	----	----	58.6 (0.6)		----	----
P	Humid	with Cu	*Bacillus sp.*	----	----	----	----	58.2 (3.9)	0.0115	----	----
		Control	*Bacillus sp.*	----	----	----		90.3 (8.0)		----	----
	Dry	with Cu	*Bacillus sp.*	----	----	53.7 (2.3)	0.0063	92.3 (7.4)	0.0141	64.7 (1.3)	0.0027
		Control	*Bacillus sp.*	----	----	26.7 (1.6)		51.3 (3.3)		55.9 (1.3)	

4. Discussion

In present study it was showed that exposure to Cu concentrations as found at the polluted field station of the river Tumbes produce a different response with respect to the field station that presents low metals pollution. Bacterial community associated with biofilm structure was affected by metal exposure and the response of taxa in RT generated a total change of diversity over time. On the other hand the diversity of taxa in P showed some bacterial species persistence over time (Figure 2 and 3).

The succession trends in the performed experiments matched in general with the observed differences in microbial communities at the reference and polluted river stations, but a simple scheme of interaction with Cu is insufficient to explain the present findings. Ivorra (2000) has previously demonstrated that the stage of biofilm maturation strongly influence community sensitivity to metal exposure. It is hypothesized here that an additional explanation for deviations from a simple metal interaction scheme may be found in the growth form and location of the individual species in the biofilm. Therefore, the proliferation of early colonising taxa, such as *Pseudomona* and *Bacillus* under Cu polluted conditions found in the present study could be partly attributed to their small size, firm fixation and rapid replication, but also by their response in terms of total antioxidant capacity after copper treatment (Cha and Cooksey 1991, Yang *et al*., 1993). Other important species found was *Aeromona trota* and *Aeromonas punctata*, in site RT, for the control group (*Aeromona trota*) and with copper (both *Aeromona punctata* and *Aeromona trota*). These species have been described habiting polluted estuarine water by Almeida and Nunes (1995). Others studies reported plasmids that codify for resistance against antibiotics and metals like arsenite and mercury in *Aeromonas* (Huddleston *et al.,* 2006; Matyar *et al.* 2010). It was observed that the

number of taxas increased during the succession, but the number of taxas decrease between treatments with and without copper (Figure 2 and 3), and this tendency has been described by Boivin *et al*. (2006) and Peterson (1996). The high heterogeneity and organic matter content as well as the lack of proper reference locations challenged the determination of effects of heavy metals on water bacterial communities. Actually selection favors bacterial communities adapted to a wide spectrum of changes in heterogeneous environments and so far, there is no information on the potential role of bacterial interactions response to metals.

Laboratory metal exposure of biofilms arising from the metal polluted stream was assumed to present lower changes, because the experimental metal concentrations was derived from the *in situ* levels. Indeed, growing biofilms coming from RT site were not inhibited in the first week and bacterial growth during copper exposure in the laboratory. Combined observations of Admiraal *et al.* (1999) and Lehmann *et al.* (1999) on the inhibition of bacterial metabolism and photosynthetic activity of benthic assemblages in the field, indicate tolerance of the assemblages in polluted streams. Several reports by Blanck *et al.* (1988), Paulsson *et al.* (2000), Soldo and Behra (2000) demonstrated that pollution can induce community tolerance (PICT) through genetic adaptation or species succession.

As mentioned above, diversity analysis revealed groups of bacteria species to be associated to polluted or un-polluted environments like *Aeromonas trota*, a specific species of polluted environmental, while other species/genus proved to be indifferent like *Pseudomonas fluorescens* and *Bacillus sp*. Interspecific and intraspecific

differences in metal tolerance, could explain the distribution of bacteria species in the field communities (Baath *et al*., 1992; Lemke, *et al*., 1996; Davis, *et al*., 2004).

In conclusion, this study demonstrated that biofilms from a non-impacted environment were more severely affected by copper exposure during an early developmental stage of development. Contrary to this, mature biofilms are highly resistant to metals even without a pre-exposure history, where diversity changes can be regarded as a selective process of tolerant species in order to maintain biofilm functionality. Still, the communities exposed in the field to very high metal concentrations contained species with known metal tolerance like *Aeromonas trota* and *Aeromonas punctata*. Metal exposure of communities coming from non-impacted environments induced changes in the species composition, suggesting selection for metal tolerance. On the other hand this ability to tolerate copper metal pollution may be being mediated by total antioxidant capacity modulation in *Pseudomonas* and *Bacillus* genera, which are two genders persistent in biofilm formation process as shown in the results (Figure 2 and 3).

It is noteworthy that the antioxidant capacity evaluated parameter is not critical and / or sufficient to establish tolerance mechanisms in these bacterial communities. However it is a comprehensive indicator of the effect ficiologico following an alteration in diversity bacterial community that integrates evaluated.

Acknowledgments

The research was supported by a Doctoral fellowship from FINCyT-LASPAU agency Authors wish to thank J.Marticorena for his invaluable logistic support. We would like to thank Tumbes University and Jorge Echevarria and INRENA for kindly supplying information from the studie sites. J. M. Monserrat is a productivity research fellowship from the Brazilian Agency CNPq (process PQ 306027/2009-7).

References

Admiraal W., Blanck H., Buckert-de Jong M., Guasch H., Ivorra N., Lehmann V., Nyström B.A.H., Paulsson M., Sabater, S. (1999). Short-term toxicity of zinc to microbenthic algae and bacteria in a metal polluted stream. *Water Research*, 33: 1989-1996.

Almeida V., Nunes, M. (1995). Presence of *Aeromonas trota* in aquatic environment. *Memórias Instituto Oswaldo Cruz,* 90: 449-451.

Anderson N.J., Rippey B., Gibson, C.E. (1993). A comparison of sedimentary and diatom-inferred phosphorus profiles: Implications for defining pre-disturbance nutrient conditions. *Hydrobiologia,* 253: 357-366.

Baath, E., Frostegard, A., Fritze, H. (1992). Soil bacterial biomass, activity, phospholipid fatty acid pattern, and pH tolerance in an area polluted with alkaline dust deposition. *Applied and Environmental Microbiology*, 12: 4026-4031.

Barranguet, C., van den Ende, F.P., Rutgers, M., Breure, A.M., Greijdanus, M., Sinke, J.J., Admiraal, W. (2003). Copper-induced modifications of the trophic relations in riverine algal-bacterial biofilms. *Environmental Toxicology and Chemistry*, 22: 1340–1349.

Beveridge T.J. and Fyte W.S. (1985). Metal fixation by bacterial cell walls. *Canadian Journal of Earth*, 22: 1893-1898.

Blanck H., Wängberg S-Å., Molander, S. (1988). Pollution induced community tolerance-A new ecotoxicological tool. *In* Cairns, J.Jr., Pratt, J.A. (eds) *Functional testing of aquatic biota for estimating hazards of chemicals. ASTM STP 988*. *American Society for testing and materials, Philadelphia*, USA, pp. 219-230.

Blum, J.L. (1957). An ecological study of the algae of the saline river, Michigan. *Hydrobiologia*, 9: 361-408.

Boivin, E.Y., Massieux, B., Breure, A.M., Greve, G.D., Rutgers, M., Admiraal, W. (2006). Functional recovery of biofilm bacterial communities after copper exposure. *Environmental Pollution*, 140: 239-246.

Boivin, M.Y., Massieux, B., Breure, A.M., van den Ende, F.P., Greve, G.D., Rutgers, M., Admiraal, W. (2005). Effects of copper and temperature on aquatic bacterial communities. *Aquatic Toxicology*, 71: 345–356.

Brookes P.C. (1995). The use of microbial parameters in monitoring soil pollution by heavy metals. *Biology Fertility Soils*, 19: 269-279.

Boor KJ (2006). Bacterial stress responses: What doesn't kill them can make them stronger. *PLoS Biology*, 4: e23.

Boles, B.R., Thoendel, M., and Singh, P.K. (2004). Self-generated diversity produces "insurance effects" in biofilm communities. *Proceeding of the National Academy of Sciences USA*, 101: 16630–16635.

Bray, J.R., Curtis J.T. (1957). An ordination of the upland forest communities of southern Wisconsin. *Ecological Monographs*, 27: 325–349.

Cha, J.-S., Cooksey, D.A. (1991). Microbiology copper resistance in *Pseudomonas syringae* mediated by periplasmic and outer membrane proteins (copper-binding proteins/blue copper proteins). *Proceeding of the National Academy of Sciences USA*, 88: 8915-8919.

Clarke K.R., Warwick R.M. (1994). Change in marine communities: An approach to statistical analysis and interpretation. *Natural Environmental Research Council*, Plymouth, UK, 234p.

Davis, M.R. H., Zhao, F.-J., McGrath, S.P. (2004), Pollution-induced community tolerance of soil microbes in response to a zinc gradient. *Environmental Toxicology and Chemistry*, 23: 2665-2672.

Ferris, F.G. (1990). Immobilization and mineralization of metallic ions by bacteria. *Energy Sources,* 12: 371-375.

García-Meza, J.V., Barrangue, C., Admiraal, W. (2005). Biofilm formation by algae as a mechanism for surviving on mine tailings. *Environmental Toxicology and Chemistry*, 24: 573–581.

Giller, K.E., Witter, E. McGrath, S.P. (1998). Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: A review. *Soil Biological Biochemistry,* 30: 1389-1414.

Hader D.P., Kumar H.D., Smith R.C., Worrest, R.C. (2007). Effects of solar UV radiation on aquatic ecosystems and interactions with climate change. *Photochemical Photobiological Sciences,* 6: 267–285.

Huddleston J., Zak J., Jeter, R. (2006). Antimicrobial susceptibilities of *Aeromonas* spp. isolated from environmental sources. *Applied and Environmental Microbiology*, 72: 7036–7042.

Ivorra N., Bremer S., Guasch H., Kraak, M.H.S., Admiraal, W. (2000). Differences in the sensitivity of benthic microalgae to Zn and Cd regarding biofilm development and exposure history. *Environmental Toxicology and Chemistry*, 19: 1332-1339.

Lehmann V., Tubbing G.M.J., Admiraal, W. (1999). Induced metal tolerance in microbenthic communities from three lowland rivers with different metal loads. *Archives of Environmental Contamination and Toxicology*, 36: 384-391.

Lemke, M.J., Brown, B.J., Leff, L.G. (1996). The response of three bacterial populations to pollution in a stream. *Microbial Ecology*, 34: 224-231.

Lu, Z.H., Cobine, P., Dameron, C.T., Solioz, M. (1999). How cells handle copper: A view from microbes. *Journal of Trace Elements in Experimental Medicine*, 12: 347-360.

Matyar, F., Akkan, T., Uçak, Y., Eraslan, B. (2010). *Aeromonas* and *Pseudomonas*: antibiotic and heavy metal resistance species from Iskenderun Bay, Turkey (northeast Mediterranean Sea). *Environmental Monitoring Assessment*, 167: 309–320.

Mitchell A., Romano, G.H., Groisman, B., Yona, A., Dekel, E., Kupiec, M., Dahan, O., Pilpel, Y. (2009). Adaptive prediction of environmental changes by microorganisms. *Nature*, 460: 220-224.

Rastogi R.P., Kumar, R.A., Tyagi, M.B., Sinha, R.P. (2010). Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair. *Journal of Nucleic Acids*, 2010: 1-32.

Palomino, J.C., Martin, A., Camacho, M., Guerra, H., Swings, J., Portaels, F. (2002). Resazurin microtiter assay plate: Simple and inexpensive meted for detection of drug

resístanse in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy,* 46: 2720–2722.

Peterson, C.G. (1996). Mechanisms of lotic microalgal colonisation following space-clearing disturbances acting at different spatial scales. *Oikos,* 77: 417-435.

Paulsson, M., Nyström, B., Blanck, H. (2000). Long-term toxicity of zinc to bacteria and algae in periphyton communities from the river Göta Älv, based on a microcosms study. *Aquatic Toxicology*, 47: 243-257.

Rice-Evans, C., Miller, N. (1994). Total antioxidant status in plasma and body fluids. *Methods in Enzymology,* 234: 279-293.

Ritchie, L.E., Steiner, J.M., Suchodolski, J.S. (2008). Assessment of microbial diversity along the feline intestinal tract using 16S rRNA gene analysis. *Microbiological Ecology*, 66: 590–598.

Serra, A., Guasch, H., Admiraal W., van der Geest, H.G., van Beusekom, S.A.M. (2010). Influence of phosphorus on copper sensitivity of fluvial periphyton: The role of chemical, physiological and community-related factors. *Ecotoxicology*, 19:770–780.

Shiu, C.T., Lee, T.M. (2005). Ultraviolet-B-induced oxidative stress and responses of the ascorbate-glutathione cycle in a marine macroalga *Ulva fasciata*. *Journal of Experimental Botany,* 56: 2851-2865.

Sobczack, W.V. (1996). Epilithic bacterial responses to variations in algal biomass and labile dissolved organic carbon during biofilm colonization. *Journal of the North American Benthological Society*, 15: 143-154.

Sokal, R. and Rohlf, F.J. (1981). Biometry. 2nd Ed. WH. Frelman & Co., San Franciasco. 859 pp.

Soldo, D., Behra, R. (2000). Long-term effects of copper on the structure of freshwater periphyton communities and their tolerance to copper, zinc, nickel and silver. *Aquatic Toxicology*, 47: 181-189.

Srivastava, A.K. (2010). Assessment of salinity-induced antioxidative defense system of diazotrophic cyanobacterium *Nostoc muscorum*. *Journal of Microbiology and Biotechnology*, 20: 1506-1512.

Stevenson, R.J., Pan, Y. (1999). Assessing environmental conditions in rivers and streams with diatoms; Pages 11-40 *in* Stoermer, S.; Smol, J. P. (editors). *The Diatoms: Applications for the Environmental and Earth Sciences*; Cambridge University Press, Cambridge, UK.

Stoermer, E.F., Smol, J.P., eds., (1999). The diatoms; applications for the environmental and earth sciences: Cambridge, U.K., Cambridge University Press, 469 p.

van Dam, H., Mertens, A., Sinkeldam, J. (1994). A coded checklist and ecological indicator values of freshwater diatoms from the *Netherlands Journal of Aquatic Ecology*, 28: 117-133.

Wetzel, R.G. (1993). Microcommunities and microgradients: Linking nutrient regeneration, microbial mutualism, and high sustained aquatic primary production. *Netherlands Journal of Aquatic Ecology*, 27: 3-9.

Yang, C-H., Menge, J., Cooksey, D.A. (1993). Role of copper resistance in competitive survival of *Pseudomonas fluorescens* in soil. *Applied and Environmental microbiology*, 59: 580-584.

- CAPÍTULO 2 -

The influence of ambiental factors on biofilm dynamics after copper exposure

Rafaela Elias Letts[1,2*], Paola Yurivilca[2], Silvia Sato Soto[2] , José M. Monserrat[1,3]

Manuscrip send to Microbial Ecology

[1]Programa de Pós-Graduação em Ciências Fisiológicas - Fisiologia Animal Comparada, Universidade Federal de Rio Grande – FURG, Rio Grande, Brazil.

[2] Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Lima, Perú

[3]Instituto de Ciências Biológicas (ICB), Universidade Federal do Rio Grande - FURG

* Corresponding author

Phone: +51 1 2714901

E-mail address: rafaela.elias@upch.pe (Rafaela Elias Letts);

Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia-UPCH, PoBox 4314, Lima-100, Perú

Resumo

Estrutura da comunidade bacteriana e sua relação com variáveis ambientais, como temperatura, pH, nutrientes e fatores antrópicos, como a poluição cobre nas comunidades microbianas na superfície dos sedimentos de ecossistemas de mangue tropicais foi investigada em amostras de Tumbes, Peru (site RT: água doce e de alta impactados com metais; P site: águas estuarinas e de baixo impacto com metais). Desenvolvimento das comunidades foram seguidos em laboratório em microcosmos durante 4 semanas, simulando as condições ambientais e de poluição para estações secas e úmidas. Perfis das comunidades bacterianas foram geradas a partir da cultura independente de PCR-electroforese em gel desnaturante (SDS-PAGE), e os resultados foram interpretados com análise estatística multivariada. análise filogenética foi utilizada para identificar a estrutura das comunidades bacterianas. Os resultados da análise de componentes principais (ACP), revelou que a concentração de metais dos sedimentos foram responsáveis por uma quantidade significativa de variabilidade na composição da comunidade bacteriana. Amostras coletadas de RT mostrou comunidades microbianas mais semelhantes entre os tratamentos com cobre (com ou sem) e nutrientes (baixo e alto nível). Em amostras de P, a semelhança entre as comunidades cluster foi apenas cobre exposta, onde os níveis de nutrientes influenciaram as comunidades de controle. Em estações de ambas as RT e P, nenhuma similaridade foi encontrada entre os controles e do biofilme de referência (levado diretamente para o campo). As diferenças de temperatura exerceu uma influência significativa na composição da comunidade em estações úmidas e secas, onde as comunidades distintas desenvolvido em 20 e 25 ^{o}C. Os resultados mostraram alterações na composição da comunidade entre diferentes tratamentos, com os conjuntos de similaridade para tratamento de cobre em ambos os sítios de RT e.

Palavras-chave: biofilme, poluição, cobre, nutrientes, temperatura, a comunidade de bactérias.

Abstract

Bacterial community structure and its relationship with environmental variables like temperature, pH, nutrients and anthropogenic factors like copper pollution on microbial communities at the surface of sediments of tropical mangrove ecosystems was investigated in samples from Tumbes, Peru (RT site: fresh water and high-impacted with metals; P site: estuarine water and low-impacted with metals). Communities development were followed in laboratory in microcosm experiments during 4 weeks, simulating environmental conditions and pollution for dry and humid seasons. Profiles of bacterial communities were generated using culture-independent PCR-denaturing gel electrophoresis (SDS-PAGE), and the results were interpreted with multivariate statistical analysis. Phylogenetic analysis was used to identify the structure of the bacterial communities. Results of principal components analysis (ACP) revealed that the metal concentration of the sediments accounted for a significant amount of the variability in the bacterial community composition. Samples collected from RT showed more similar microbial communities between the treatments with copper (with or without) and nutrients (low and high levels). In samples from P, the cluster similarity was between only copper-exposed communities, where nutrient levels influenced the control communities. At both RT and P stations, no similarity was found between controls and the reference biofilm (taken directly for the field). Temperature differences exerted a significant influence on community composition in humid and dry seasons, where distinctive communities developed at 20 and 25 ^{o}C. The results showed changes in communities composition between different treatments, with clusters of similarity for copper treatment in both RT and P sites.

Key words: biofilm, copper, pollution, nutrient, temperature, bacteria community

1. Introduction

In mangrove systems, the great variation in microbial activity and diversity observed during a single summer probably exceeds that of the most dramatic successions of terrestrial plant communities spanning many years (Wetzel, 1983; Giller *et al*., 1994). In the course of a few days, the habitat of a microbial community may shift from hipersaline to low salinity or oxygen supersaturation to anoxia; from an excess of dissolved nutrients to complete nitrogen depletion (Van Dam & Mertens, 1995; Borchardt, 1996). New microbial niches will be created, filled and destroyed in rapid succession and season changes during a natural cycle. The net result of all physical chemical microbial interactions is the way the ecosystem functions, usually characterized by the patterns and scale of mineral cycling and carbon fixation, meaning that microbial activity is inseparable from ecosystem function (Fenchel *et al*., 1999; Raymond, 2005). This idea can be extended to state that also microbial diversity is inextricably linked to ecosystem function, if assumed that all microbial niches are always filled. Reciprocal interactions between microbial activity and the physical chemical environment create a continuous turnover of microbial niches that are always filled, so microbial diversity is a part of ecosystem function. The rapid niches turnover is favoured because microbes are inherently fast growing, extremely abundant, easily dispersed over large distances, and unlikely to become locally extinct (Fenchel, 1993; Larsen *et al*., 2007). So, all new microbial niches created are likely to be filled within a short time by recruitment from the locally available diversity of rare and dormant microbes (Finlay *et al.*, 1996; Fenchel *et al*., 1997). If new microbial niches are always filled, the number of microbial active species at any point in time (i.e., the microbial diversity) depends upon the number of microbial niches available which, in turn, depends upon the interaction between microbial activity and the physico-chemical

environment. We hypothesize that the species complement of the microbial community quickly adapts, even to transient changes in the local environment.

Mangrove ecosystems are dominant ecosystems along tropical coastlines and they have important relationships with the regulation and optimisation of tropical marine environments. They are thought to be very important as primary producers of organic matter, providing the base for a large and complex food web (Riemann *et al*., 2000; Farooq & Malfatti, 2007). Although mangrove ecosystems are rich in organic matter, in general they are nutrient deficient, especially with regard to nitrogen and phosphorus (Sengupta and Chaudhuri, 1991; Holguin *et al.,* 1992; Alongi *et al.,* 1993; Vazquez *et al.,* 2000; Farooq & Malfatti, 2007), where microbial activity is responsible for major nutrient transformations within a mangrove ecosystem (Alongi *et al.,* 1993; Holguin *et al.,* 2001). In Peru, mangrove ecosystems like that of Tumbes are seriously polluted by heavy metals like copper (CDC, 1992; CART, 2002). It has been documented that microorganisms play an important role in the productivity, conservation and rehabilitation of mangrove ecosystems (Holguin *et al.,* 2001). Thus, knowledge of microbial community diversity and the relationship between environmental factors and structure of bacterial communities in mangrove sediments is important for understanding how the mangrove ecosystems function, an important aspect to formulate effective management and conservation strategies. To understand the community and diversity of bacteria in mangrove ecosystems, and the relative influences of stressful factors like copper pollution on dynamic change of microbial species, we employed a microcosmos assay. It was evaluated during four weeks the biofilm formation and the structure of communities was recognized with molecular techniques based on PCR 16S rRNA evaluated in SDS-PAGE (Li *et al.*, 2006; Liang *et al.*, 2007; Muckian *et al.*,

2007). The environmental parameters tested were temperature, pH, nutrient (NKP), UVB and copper.

2. Materials and methods

2.1. Analysis of water and mud

The characteristic of water and mud collected in the field was determinated two times by year. Parameters tested were dissolved oxygen concentration, pH, temperature, conductivity and solid suspension, determined *in situ* with a multiparametric equipment (WTW). Nitrate, phosphate and potassium concentration were measured by colorimetric kits (HANNA, model HI 83225 Grow Master) and UVB intensity was determined with a Luxometer (Delta OHM). Metal concentration in water and mud was analyzed through Optic-ICP. All readings were made in triplicate.

2.2. Test site and sampling

The mangrove areas studied are located at the mouth of the Tumbes River, in Peru (03º25´LS and 80º17´LW) (Fig. 1). Two sites were analyzed: Tumbes River (RT), extremely polluted by a former polymetalic mine (CDC, 1992; CART, 2002), whereas a fishing station (P) at the Faculty of Fishing, Tumbes National University was considered a reference site, according to preliminary determination of metal levels.

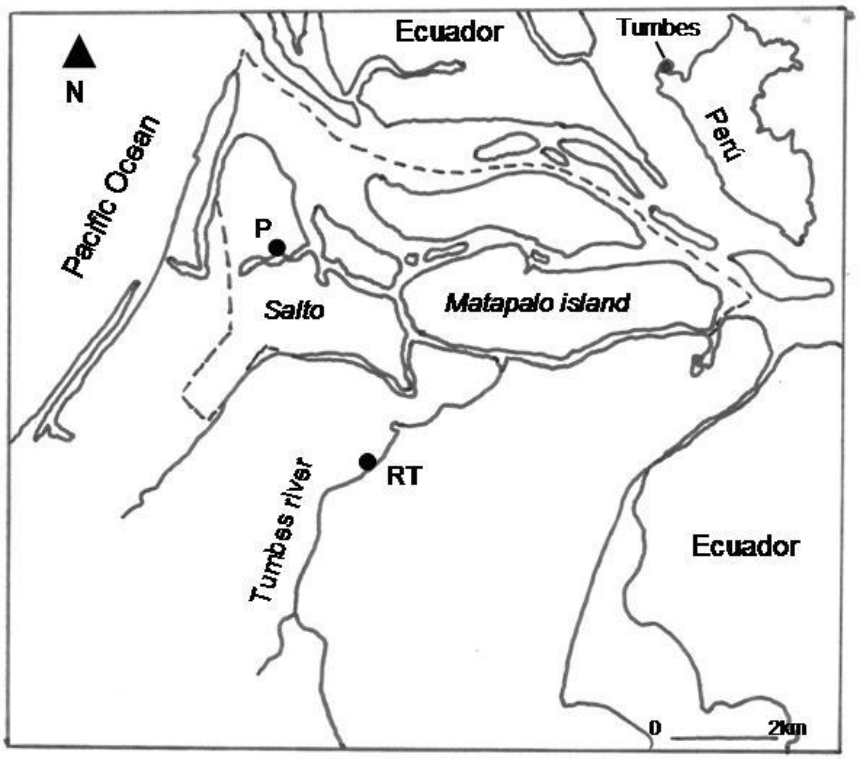


Figure 1. Location of the experimental sites in Tumbes mangrove: the polluted site RT (Tumbes river; 17560527E 9605246N UTM) and the reference site P (Faculty station model 17567257E - 9612703N UTM).

2.3. Biofilm production

Glass slides (7.5 x 2.5 cm^2 surface) were used as an artificial and selective substratum for bacterial attachment. Before use, glass slides were soaked in 10% HNO_3 (grade reagent) solution for 24 h and then rinsed with deionized water. The glass slides were placed inside a polyethylene tank with 50 L de mangrove water sampled from the two sites. Each tank supported 70 slides, and it was provided with a water recirculation system. Several treatments were established in order to evaluate the influence of ambiental factors like temperature, pH, nutrients and copper on the development and dynamics of bacterial communities associated to biofilm (Boivin *et al*., 2005). By this

way, two temperatures (20 and 25ºC); two potassium concentration (3.5 and 22.0 mg/L), three phosphorus concentrations (0.5; 1.5; and 110.0 mg/L) and three nominal copper concentrations (P site 100 or 200 mg/L; RT site 200 or 400 mg/L) were selected in virtue that these values represent mean values registered between humid and dry season (February and July respectively), finally it was included a nutrien composition of humid season (potassium 3.5 and phosphorus 0.5 mg/L) we will call NA and NB for dry season (potassium 22.0 and phosphorus 110.0 mg/L). After 4 weeks of exposure, 3 glass slides were removed for each tank and a sample of the early biofilm was taken for bacterial growing in nutritive medium (Merck), with constant shaking during 24 h at the same temperature of exposure in each tank. The cultures were centrifuged and the pellet stored at -80ºC until DNA extraction.

2.4. Extraction of DNA

Each bacteria pellet obtained as described previously, was extracted and analysed separately. DNA extraction was carried out using a bead-beating method, followed by phenol:chloroform:isoamylalcohol (25:24:1 v/v) extraction (Suchodolski *et al*., 2008).Purified DNA was stored at -80 ºC until further use. A negative control containing H_2O instead of a sample was purified in parallel to each extraction batch in order to screen for contamination of extraction reagents. 16S rRNA gene amplification by PCR of extracted DNA was used as a template for PCR amplification of 450-bp amplicon of the 16S rRNA gene with universal bacterial primers (final concentration: 0.25 µM for each primer): F-341 (5'-CCTACGGGAGGCAGCAG-3') and R-786 (5'-GACTACCAGGGTATCTAATC-3') (Ritchie *et al*., 2008). Reactions were performed with the kit HotStart HiFidelity (Qiagen), using 60 ng of DNA template. To screen for potential contamination of PCR reagents, a negative PCR control using H_2O instead of a

DNA template was used. The samples were amplified in a thermocycler (Mastercycler Gradient, Eppendorf AG, Hamburg, Germany), using the following PCR protocol: initial denaturing at 95 ºC for 3 min, 30 cycles of denaturation at 95 ºC for 30 s, annealing at 54 ºC for 30 s, extension at 72 ºC for 1 min, and a final extension at 72 ºC for 10 min. The purity of the PCR amplicons was assessed on 1% agarose electrophoresis gels stained with ethidium bromide (Ritchie *et al*., 2008).

2.5. SDS-PAGE.

Communities diversity were monitored by SDS-PAGE with 6% urea and polyacrylamide:bisacrylamide 20:1. The samples were run at 300 V for 50 min, then at 250 V for 150 min and finally at 65 V for 10 h. Gels were stained with ethidium bromide and digital gel images obtained for analysis using Phoretix 1D software (Nonlinear Dynamics, Newcastle, United Kingdom).

Images were analyzed as follows: lanes were recognized automatically by program. Each lane represented only one sample. First, automatic band detection was performed with a minimum slope of 75 to 100 and a noise reduction of 5, and peaks smaller than 2% of the maximum peak were discarded. The edge detection method was fixed to a width of 1. Then bands were assessed and corrected by eye, one by one. Bands were then matched to create a matrix containing band intensity, molecular weight (MW) and number of bands for line respect to MW (low range 100 bp Bio Rad) line values of each sample. The band matrix was subjected to non-metric multidimensional scaling (MDS) (Field *et al*., 1982).

2.6. Measurements of biochemical variables

Total antioxidant capacity was measured with a methodology adapted from Rice-Evans *et al*. (1994), using 2,2-azinobis(3-ethylbenzothiazoline 6-sulfonate) reagent (DPPH). Each CFU from control and copper exposed groups were lised by sonicator for 15 min at 60 Hz (BRASONIC ultrasonic cleaner, model 3510) and measured in triplicate in 96 well plates, being registered the absorbance at 517 nm using an ELISA reader (VERSA max microplate reader). Total antioxidant capacity was estimated according to the following formulae:

$CA\% = [1- (A_2-A_3)/A_1] \times 100$,

where: A_1: Absorbance of DPPH solution alone, A_2: absorbance of DPPH solution plus the sample, and A_3: blank absorbance.

Viability assays were carried out in 96 well plates following a modified methodology described by Palomino *et al*. (2002). To 100 µL of the suspension it was added 30 µL of resazurin solution 0.01% (w/v) to each well, incubated overnight at 37 ºC and assessed for color development at 570 nm in a microplate reader (Bio-Tek Instruments). It was considered 100% of viability the mean absorbance of samples from the control group.

2.7. Statistical analyses.

To evaluate the internal structure of the data and their relationship with environmental and anthropogenic factors it was employed Principal Component Analysis (PCA). Differences between treatments were statistically tested by using a parametric factorial. The similarity matrix calculation was based on band percentage data retrieved from the gel image analysis using Phoretix 1D and was performed using the Bray-Curtis index. Similarity matrixes were used to construct non-metric multidimensional scaling (MDS)

ordinations to visually evaluate variations in the bacterial community compositions as a function of temperature, nutrients and copper at the two fixed season levels (Clarke & Warwick, 1994; Harder *et al.,* 2003). The results of the evaluation of total antioxidant capacity were analized by ANOVA.

3. Results

Metal concentration in sediment was two orders of magnitude higher at RT than in P. Is important to note that levels for Mn exceed the levels related to environmental pollution, which is 10 mg/L (OMS, 2003) (Table 1). The metal differences between the two sites, and the consequent increase in water conductivity should be related to the presence of fresh water at RT and saline/estuarine water at P site that also presented low concentration of Cu. Differences in the concentration of particles in suspension are linked to the major decomposition material in the water column of P site (Table 1). The pH in the two sites was neutral and similar.

Table 1. Mean and SD values corresponding to physical and chemical parameters of water and sediment determined during the experimental period (2009-2011) generating continuity readings during this period. Limit ISQG (Interim Sediment Quality Guideline) for copper = 35.7 mg/kg and Limit PEL (Permisible Exposure Limit) for copper = 35 mg/kg.

	Tumbes river (RT)				Fishing station (P)			
Parameter	Humid		Dry		Humid		Dry	
	Mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.
pH	7.4	0.1	7.88	0.1	7.4	0.3	7.6	0.0
Dissolved oxygen (%)	18.2	3.3	20.3	1.2	13.0	1.2	17.0	0.2
Particles in suspension (ppm)	250.0	10.8	180.0	43.1	2,000.0	12.6	2,000.0	10.8
Temperature (°C)	26.2	0.2	25.4	1.1	27.6	0.9	26.0	1.7
Conductivity (µS)	127.0	2.3	105.0	55.6	3,999.0	0.0	3,999.0	0.0
UVB (W/m^2)	435.9	91.2	370.0	24.1	17.5	1.4	16.0	2.1
Potassium (mg/L)	1.0	0.1	1.0	0.2	0.0	0.0	0.0	0.0
Cu (mg/Kg)	986.4	65.3	1,790.4	108.3	214.4	11.6	214.4	28.3
Zn (mg/Kg)	1,286.45	102.0	1,086.5	66.3	157.9	18.4	157.5	15.0
As (mg/Kg)	577.0	36.1	557.5	29.6	7.7	0.4	7.75	3.3
Pb (mg/Kg)	667.0	24.6	656.0	35.5	28.5	2.1	23.5	8.3
Mg (mg/Kg)	5,620	0.2	5312	0.23	3,050	0.12	3184	0.5
Ba (mg/Kg)	175	12.4	210	10.3	16	0.4	21	1.2
Mn (mg/Kg)	1,720	0.1	1.585	0.2	136	9.8	140	3.2

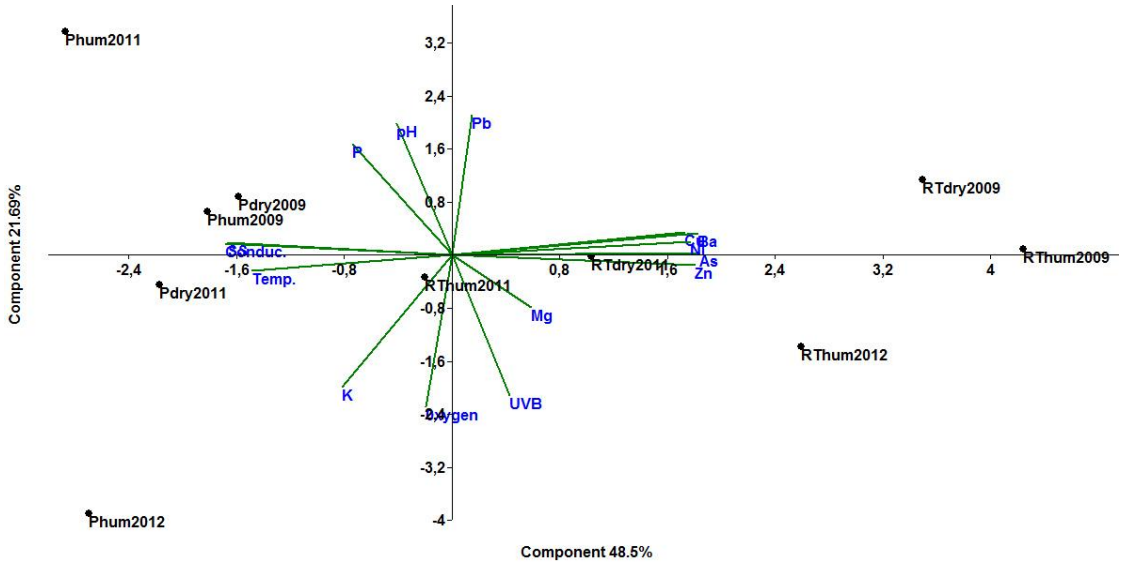


Figure 2. Principal component analysis (PCA). The horizontal axis explains 48.5% of the variance observed, while 21.69% can be explained by the vertical axis. The names of the cases were assigned by site sampling (P or RT), season (humid or dry) and the year that was done.

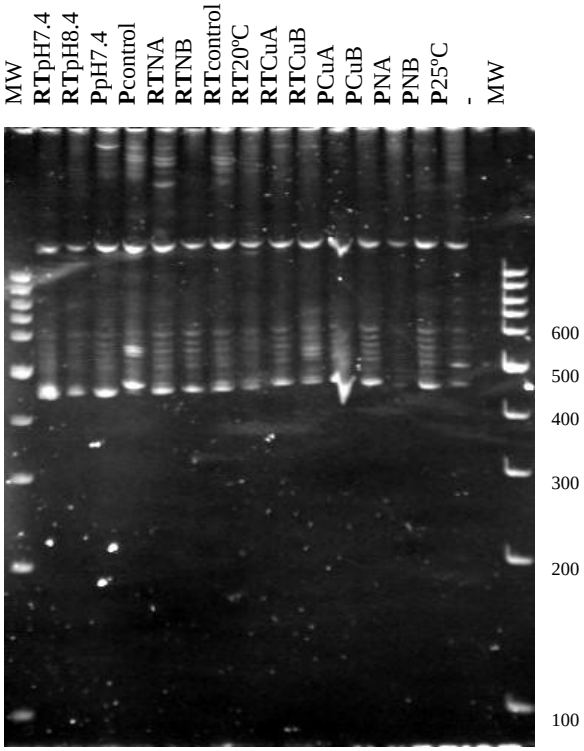


Figure 3a. PCR-PAGE of 16S rDNA banding profiles of samples collected from RT and P sites. Codes: MW (3 µl of molecular weight marker), RTpH7.4 (samples from RT incubated at pH 7.4 at 25 ^{o}C), RTpH8.4 (samples from RT incubated at pH 8.4 at 20 ºC), PpH7.4 (samples from P incubated at pH 7.4 at 20 ºC), Pcontrol (sample incubates at 20ºC, pH 7,4 and PNA nutrient concentration), RTNA (sample from RT incubated with 1 mg/L of phosphorus and 10 mg/L of potassium), RTNB (sample from RT incubated with 0.5 mg/L of phosphorus and 3.5 mg/L of potassium and pH 8.4 at 20 ºC), RTcontrol (sample incubated at 20ºC, pH 7,4 and RTNA nutrient concentration), RT20ºC (sample from RT incubated at 20 ^{o}C and pH 8.4), RTCuA (sample from RT site exposed to 400 mg/L of nominal copper at 25 ºC and pH 8.4), RTCuB (sample from RT site exposed to 100 mg/L of nominal copper), PCuA (sample from RT site exposed to 200 mg/L of nominal copper at 20 ºC and pH 8.4), PCuB (sample from RT site exposed to 10 mg/L of nominal copper at 20 ºC and pH 8.4) PNA (110 mg/L of

phosphorus and pH 7.4 at 20 ºC) PNB (sample from P incubated with 1.5 mg/L of phosphorus and 22 mg/L of potassium and pH 7.4 at 20 ºC), P25ºC (sample from P incubated at 20 ^{o}C and pH 7.4), - (negative control), MW (3 µl of molecular weight marker).

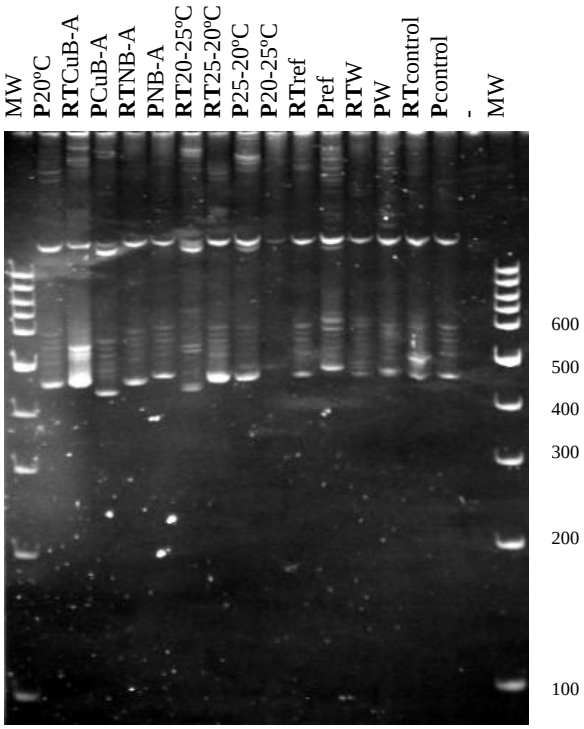


Figure 3b. PCR-PAGE of 16S rDNA banding profiles of samples collected from RT and P sites: MW(3 µL), P20ºC, RTCuB-A (100-400 mg/L of nominal copper), PCuB-A(10-200 mg/L of nominal copper), RTNB-A (0.5 and 3.5 mg/L of phosphorus and potassium, respectively– 1 and 10 mg/L of phosphorus and potassium, respectively), PNB-A (1.5 and 22 of phosphorus and potassium, respectively– 1 and 110 mg/L of phosphorus– 1) corresponding to the humid season A and B for the dry season, RT20ºC-25ºC, RT25ºC-20ºC, P25ºC-20ºC, P20ºC-25ºC, RTref, Pref., RTW (water), PW (water), RTcontrol (sample incubated at 25ºC, pH7,4 and RTNA nutrient concentration), Pcontrol (sample incubated at 25ºC, pH7,4 and PNA nutrien

concentration), - (negative control), MW (5 µL). Treatments where not specified were conducted at 20 ºC and pH 7.4 to pH 8.4 for P and RT respectively.

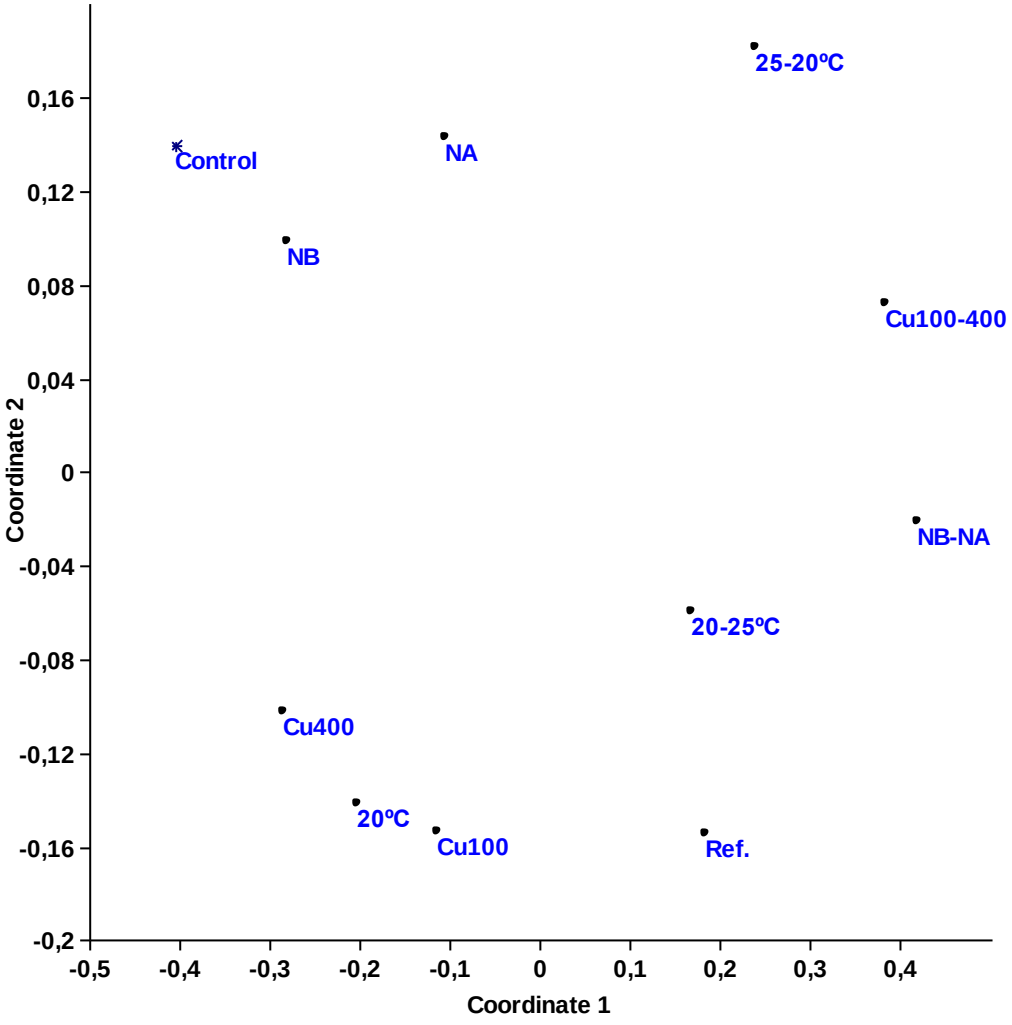


Figure 4a. Two-dimensional representations of a non-metric multidimensional scaling (MDS) of a treatment similarity of the bacterial communities exposed to copper (100 and 400 mg/L), temperature (20 and 25ºC) and nutrient NA (1 and 10 mg/L of phosphorus and potassium, respectively) and NB (0.5 and 3.5 mg/L of phosphorus and potassium, respectively), NA-NB, 25-20, 20-25ºC and Cu100-400 correspond to change

of intensity of parameter for nutrient, temperature and nominal copper respectively from RT site.

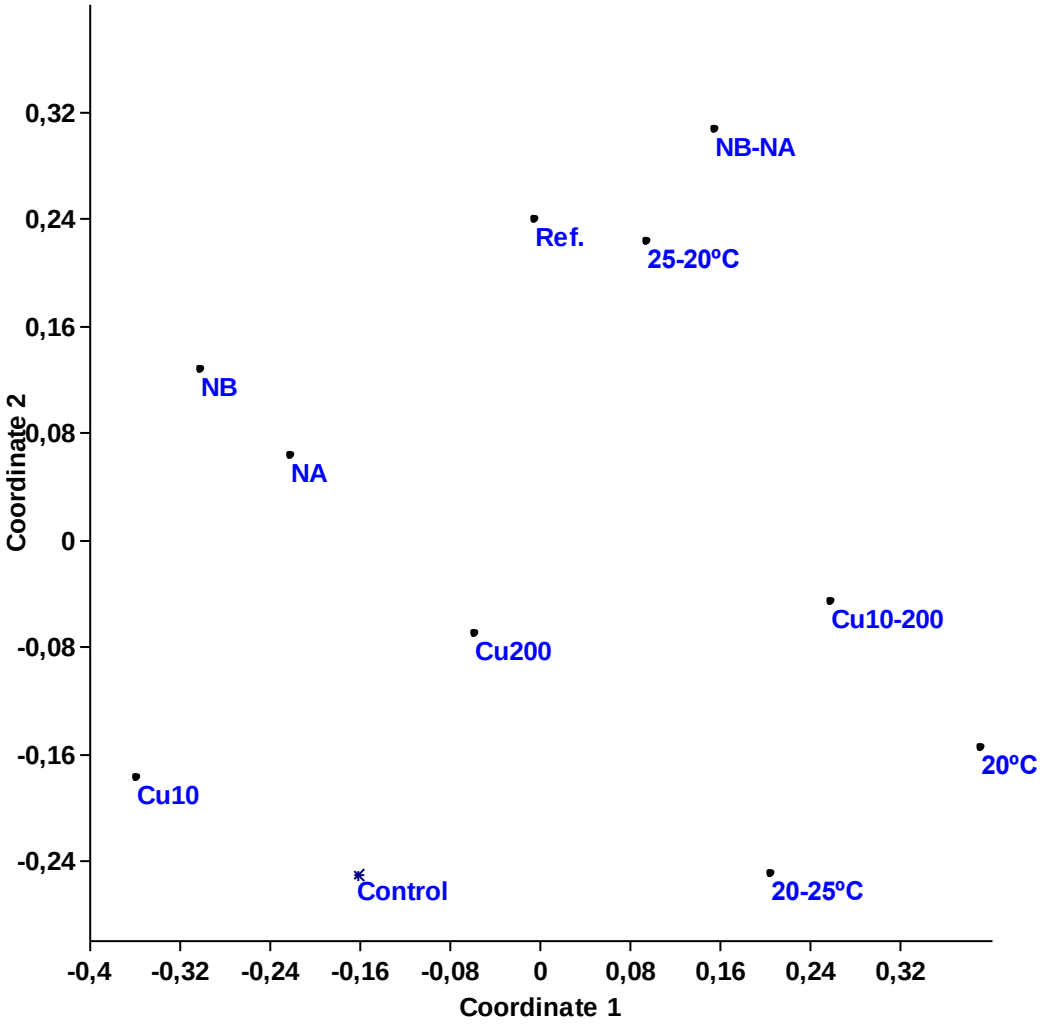


Figure 4b. Two-dimensional representations of a non-metric multidimensional scaling (MDS) of a treatment similarity of the bacterial communities exposed to nominal copper (10 and 200 ppm), temperature (20 and 25ºC) and nutrient, NA (110 mg/L of phosphors), NB (1.5 mg/L and 22 mg/L of phosphorus and potassium, respectively);

NB-NA, 25-20, 20-25ºC and Cu10-200 correspond to change of intensity of parameter for nutrient, temperature and copper respectively from P site.

The differences between bacterial community compositions in biofilms developed at different temperatures, nutrient and copper concentration were respectively visualized in a non-metric multidimensional scaling (MDS) plot (Fig. 4). As much in P as RT the reference point (biofilm taked in sampling site) are communities distant from the control point (biofilm development in the microcosms experiment), being smaller the difference in the case of P. The results from RT showed an association between the control point and the treatments with nutrients. Other groups are formed by communities generated with change treatment for copper, nutrient and temperature. In the case of P, both copper and nutrients treatments formed distant groups from both the control and reference points. In both cases (RT and P) the change treatments generated independent groups and these groups presented the highest distance from control and/or reference point (Fig. 4a, b).

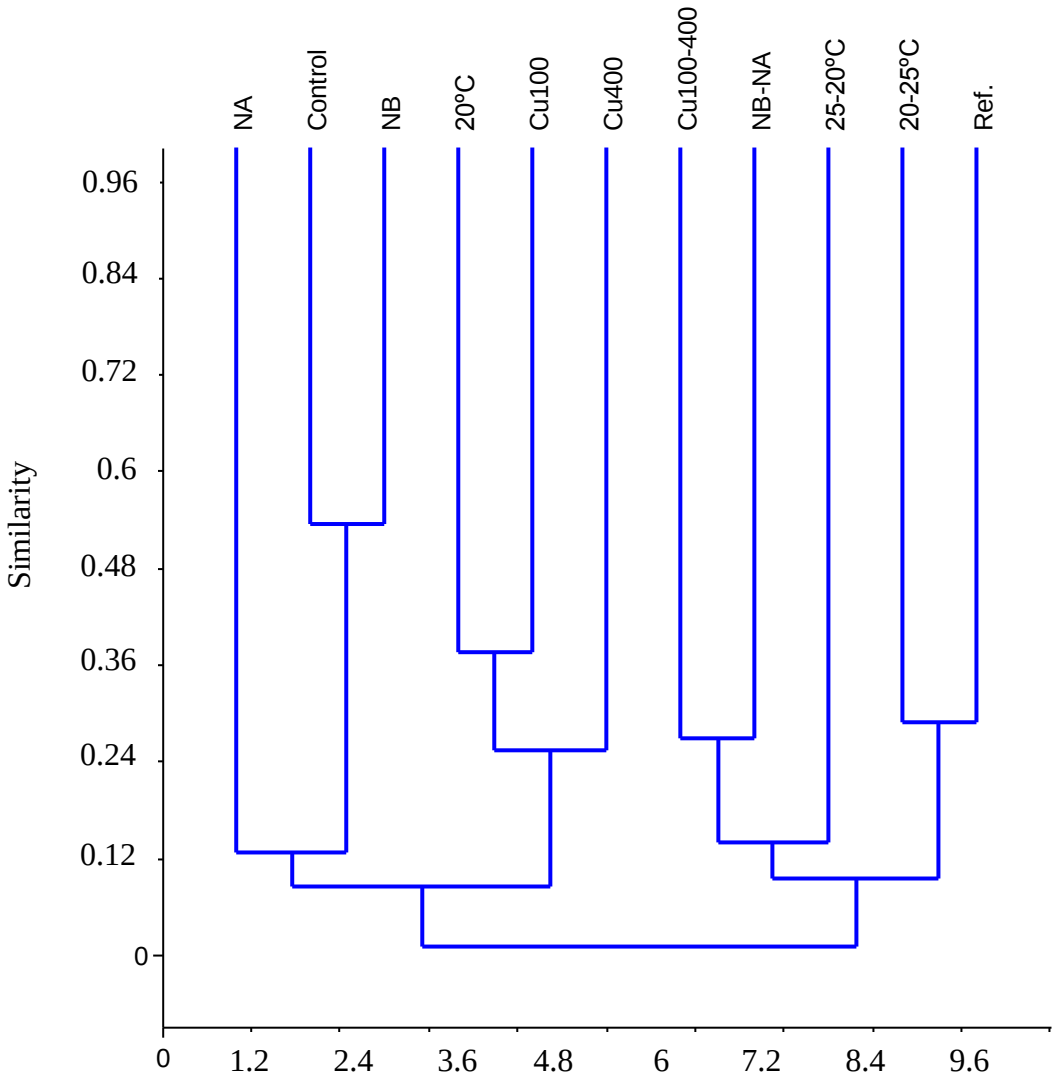


Figure 5a. Cluster analysis of 16S rDNA banding profile for bacterial communities from RT site with different treatments, where the variable to be considered was molecular weight, number of bands and band intensity. Treatments codes: NA (1 and 10 mg/L of phosphorus and potassium, respectively); NB (0.5 and 3.5 mg/L of phosphorus and potassium, respectively); Cu (100 and 400 mg/L of copper), temperature (20 and 25ºC); NA-NB, 25-20, 20-25 ºC and Cu100-400 correspond to change of intensity of parameter for nutrient, temperature and copper respectively.

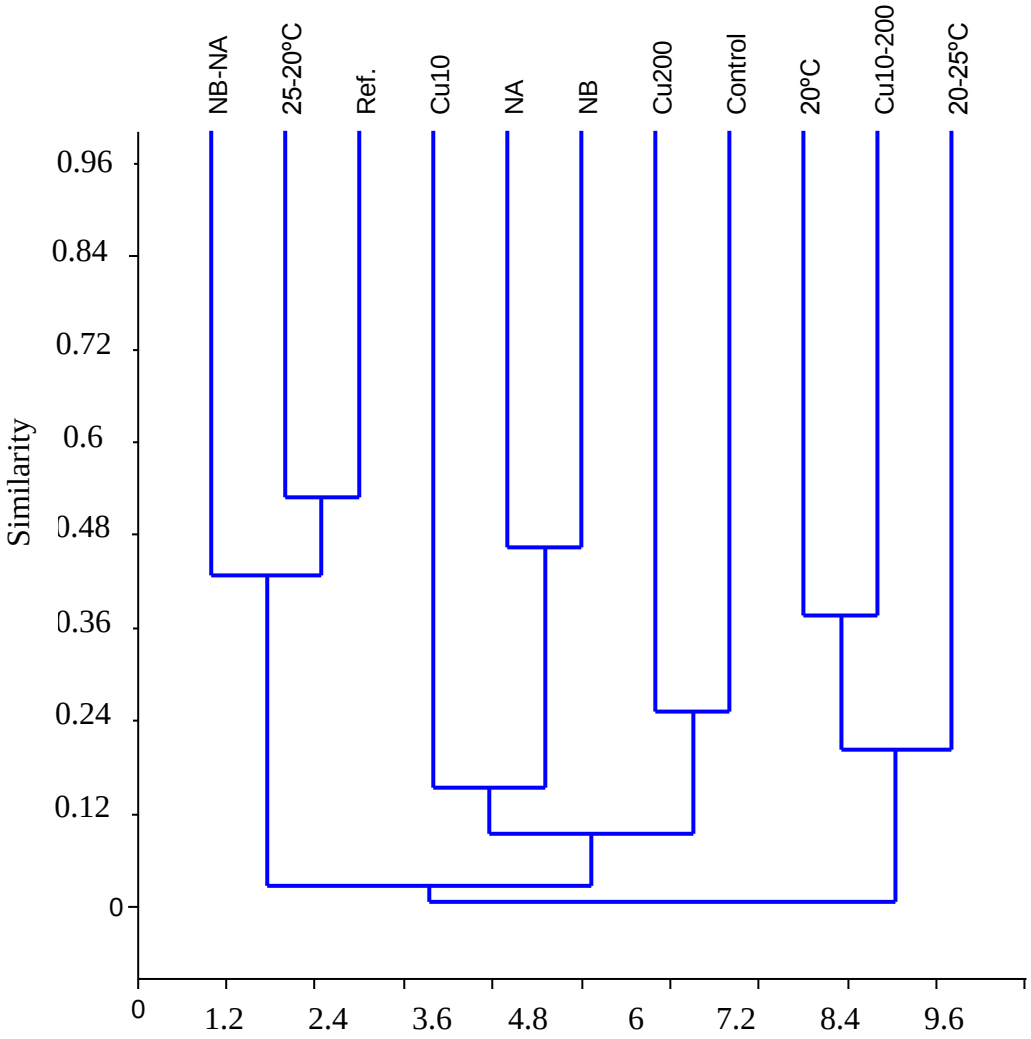


Figure 5b. Cluster analysis of 16S rDNA banding profile for bacterial communities from P site with different treatments, where the variable to be considered was molecular weight, number of bands and band intensity. Treatments codes: NA (1 and 10 mg/L of phosphorus and potassium, respectively); NB (0.5 and 3.5 mg/L of phosphorus and potassium, respectively); Cu (100 and 400 mg/L of nominal copper), temperature (20 and 25ºC); NA-NB, 25-20, 20-25ºC and Cu100-400 correspond to change of intensity of parameter for nutrient, temperature and copper respectively.

The assays did not reveal a clear relationship between communities formed by change treatment with respect to control. Moreover copper treatments are distanced from the reference samples for the P and RT sites. It is important to mention the low similarity between the control and the site of reference for P and RT sites. As much for P as for RT, the individual treatments maintained similarity with the control (Figure 5a and 5b). The results also showed proximity between control with cooper in site P and nutrient in site RT, and references points showed proximity with the communities results from change treatments for both P and RT sites (Figure 5a and 5b).

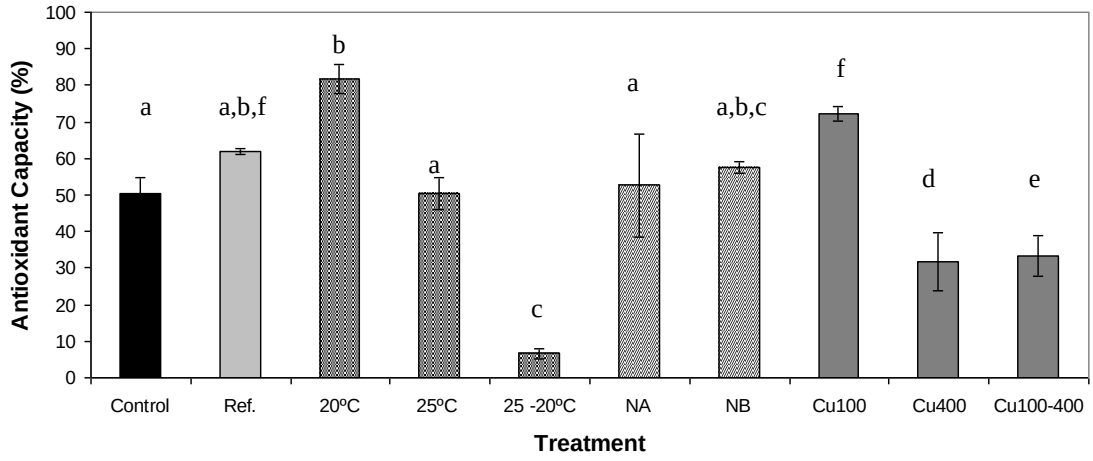


Figure 6a. Antioxidant capacity for each treatment to samples from RT site. Black bar: control; light grey: biofilm reference (Ref.); chess bar: temperature treatment 20, 25^{o}C and changes from 25 to 20^{o}C (25-20ºC); diagonal bar: NA (1 and 10 mg/L of phosphorus and potassium, respectively) and NB (0.5 and 3.5 mg/L of phosphorus and potassium, respectively); dark bar: Cu 100, 400 and 100-400 mg/L treatment. Similar letters indicate absence of statistical differences ($p>0.05$).

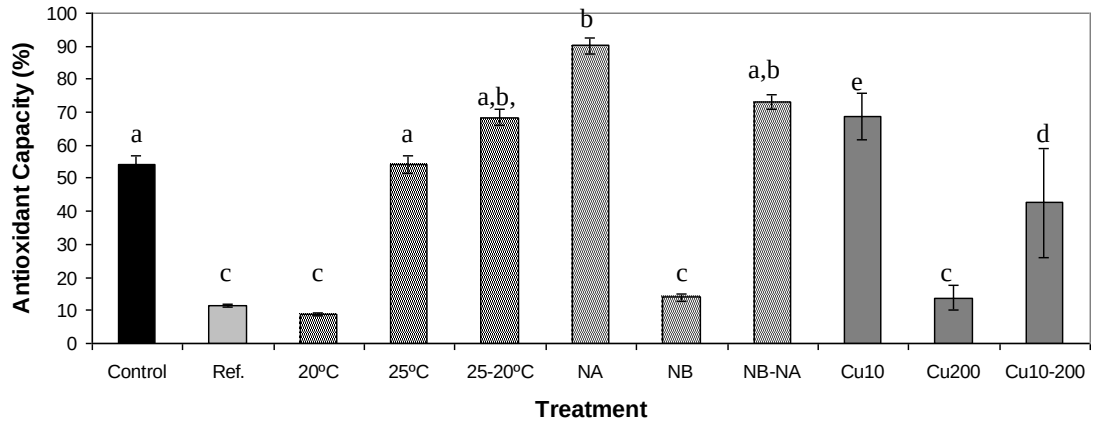


Figure 6b. Antioxidant capacity for each treatment to samples from P site. Black bar: control; light grey: biofilm reference (Ref.); chess bar: temperature treatment 20, 25 ^{o}C and changes from 25 to 20 ^{o}C (25-20 ºC); diagonal bar: NA (1 and 10 mg/L of phosphorus and potassium, respectively) and NB (0.5 and 3.5 mg/L of phosphorus and potassium, respectively); dark bar: Cu 100, 400 and 100-400 mg/L treatment. Similar letters indicate absence of statistical differences ($p>0.05$).

The total antioxidant response generated by temperature changes in P and RT are inversely proportional and consistent with the result showing their respective reference sample (taken directly from the field) river however show different antioxidant response between the P and RT sites. We observed at RT for statistical significance among the three temperature treatments, if the statistical significance of P is between one trestment of 20 ºC with 25 ºC, finding a stabilization phase in the treatment of 25-20 ºC. The results from the reference and control samples were similar for RT site and different for P. The antioxidant responses between P and RT for different treatments showed significant differences for the respective reference biofilm ($p<0.05$), temp. 20 °C ($p<0.05$), the treatment of change 25-20 °C ($p<0.05$) and nutrients NB ($p<0.05$). Finally,

one can observe an alteration in antioxidant capaciddad evaluation vs control. Cu100 and its transfer to CU400, inducing a compensatory response, which shows that compared with moderate EAOs antioxidant mechanisms are activated (Fig. 6a).

On the other hand, we observe that, irrespective of the effect of selection of bacterial diversity in the type of substrate lateracion to glass slides and efcto by the culture medium used, there is variability falata getting regain their antioxidant capacity level (fig. 6b). This can be influenced for seasonal variability drop associated mangrove leaves as an additional component supplementing antioxidant capacity (Asha *et al*., 2012).

4. Discussion

Results from this study add to the growing evidence that temperature and nutrients are important factors that regulate biofilms structure, these responses being affected by metals like copper. The mesocosmos approach employed, evaluated the bacteria community development from samples taken at Tumbes River (RT) and at a Fishing station (P), two sites with streaking differences in salinity, nutrient and metal concentration.

The observed variations in the way that the bacterial communities responded to the temperature and nutrients treatments in dry and humid seasons may be due to different prevailing bacterial propagules, presenting distinct growth and survival rates at the same temperature and salinity like reported by Admiraal *et al.* (1984) and Griffith *et al.* (1994). For instance, dry propagules may survive better and grow faster at low salinity, while winter propagules may, in contrast, survive better and grow faster at low

temperature (Griffith *et al*., 1994). Season, temperature, and salinity also influenced the antioxidant capacity of biofilms (Carvalho and Fernandes, 2010; Qurashi and Sabri, 2011). Furthermore, winter biofilms were nutritionally poorer than summer biofilms (Chénier *et al*., 2003). This agrees with an earlier field study on the nutritional quality of epilithic biofilms which demonstrated that C/N ratio peaked in winter and reduced in summer in Hong Kong (Williams *et al.,* 2000). The incorporation of nutrient was added to a final nominal concentration of 158 μg/L (1.64 μmol of P/L) to avoid nutrient depletion and P or N limitation (Bonnineau *et al*., 2010), we characterize nutrients levels in the two seasons in P and RT site. On the other hand, the measurement of antioxidant enzyme activities in biofilms is an estimation of the capacity of the whole community to respond to oxidative stress. The result of Bonnineau *et al*., (2010) showed photo-inhibition of the antioxidant enzyme catalase in algae, showing the sensitivity of this enzyme to environmental factors. On the other hand we observed a decline in the total antioxidant capacity in NA= 50% and NB= 60% for RT site respect to P site that showed values of NA= 65% and NB= 92%, however the values in treatments expossed to NB concentration shows the highest values of antioxidant capacity for both RT and P sites. Several works showed that the ratio C/N was associated to production of reactive oxygen species (ROS) and reactive nitrogen species such as NO (Foley *et al*., 2004). Similarly, Taylor *et al.* (2004) described the effects of oxidative stress on enzymes of the tricarboxylic acid cycle and showed how this might impair the carbon–nitrogen interaction, since H_2O_2 was able to inhibit citrate-stimulated O_2 consumption in mitochondria, although O_2 consumption was recovered following the addition of isocitrate. Mitochondria fulfill key roles in photosynthetic N metabolism, including the oxidation of glycine and other substrates that produce organic acids for N assimilation. (Foyer and Bowshe, 2004). Our results also imply that different community structure of

bacteria colonized the biofilms and how they change by several abiotic factors, and the impact on their functionality. In this way, variables like season, temperature, and salinity affect the bacterial community compositions, but copper was an important disturbing factor in biofilm composition. Furthermore, the biofilm has a different response depending on the characteristic of the proceeding site. The changes in bacterial denaturating PAGE profiles did show a consistent direction related to the Cu treatment. The increasing differences of the bacterial-community structures in the different treatments by site (RT and P) coincided with the development of differences in metabolic capacities as evidenced by total antioxidant capacity analysis. The proportional effect of Cu as observed here is in contrast to observations by Konstantinidis *et al*. (2003), whom used terminal restriction fragment length polymorphism to study bacteria along a depth profile in two copper-contaminated lake sediments. On the other hand, a growing number of reports suggest that, besides nitric oxide, reactive oxygen species (ROS) may be considered as key signaling molecules in living cells (Magder, 2006; Droge, 2001; Jones, 2006; Jones, 2008). Recently it was proposed that superoxide or another ROS acts as a mediator of Ras-induced cell cycle progression (Irani *et al*., 1997; Krawiec *et al*., 2000; Droge 2001; Ortiz *et al*., 2012). Krawiec (2000) showed that when osmotic shock experiments were performed in single vessels many enzymes necessary for heme synthesis are not only present, but also able to function in anaerobically grown cells, characteristic of site P that receive water from the sea and river Tumbes. Due to constant exposure to osmotic stress, communities from P site should be more prepared to tolerate these changes.

These results showed that relatively short disturbance events can alter community composition and the direction of change depends on the type of disturbance, strongly supporting the suspected role of disturbance for species composition of microphytobenthos (Peterson *et al*., 1994; Biggs and Smith 2002; Berga *et al*., 2012). The species composition after these events was diversifying only very slowly, depending on local conditions. In conclusion, the microcosmos approach provided a mechanistic framework for analyzing species composition in relation to the highly dynamic processes of growth and disturbance.

Acknowledgments

The research was supported by a Doctoral fellowship from FINCyT-LASPAU agency Authors wish to thank C. Mere for his invaluable logistic support. We would like to thank Tumbes University and Jorge Echevarria and INRENA for kindly supplying information from the study sites. J.M. Monserrat receives a productivity research fellowship from the Brazilian agency CNPq (process number PQ 306027/2009-7)

References

Admiraal W., Peletier H., Brouwer T. (1984). The seasonal succession patterns of diatom species on an intertidal mudflat: an experimental analysis. *Oikos,* 42: 30-40.

Alongi, D.M., Christoffersen, P., Tirendi, F. (1993). The influence of forest type on microbial-nutrient relationships in tropical mangrove sediments. *Journal of Experimental Marine Biology and Ecology,* 171: 201-223.

Asha K.K. Suseela Mathew & P.T. Lakshmanan. (2012). Flavonoids and phenolic compounds in two mangrove species and their antioxidant property. Indian Journal of Geo-Marine sciences, Vol. 41(3), pp 259-264.

Berga M., Szekely A.J., Langenheder S. (2012). Effects of disturbance intensity and frequency on bacterial community composition and function. *PloS ONE* 7: e36959. doi:10.1371/journal.pone.0036959.

Biggs, B.J.F. (1996). Patterns in benthic algae of streams. In *Algal ecology: Freshwater benthic ecosystems* (Eds Stevenson, R.J., Bothwell, M.L. and Lowe, R.L.), Academic Press, San Diego, p 31-56.

Biggs, B.J.F., Smith, R.A. (2002). Taxonomic richness of stream benthic algae: Effects of flood disturbance and nutrients. *Limnology Oceanography,* 47: 1175-1186.

Boivin, E.Y., Massieux, B., Breure, A.M., Greve, G.D., Rutgers, M., Admiraal, W. (2005). Functional recovery of biofilm bacterial communities after copper exposure. *Environmental Pollution,* 140: 239-246.

Bonnineau, C., Bonet, B., Corcoll, N., Guasch, H. (2010). Catalase in fluvial biofilms: a comparison between different extraction methods and example of application in a metal-polluted river. *Ecotoxicology,* 20, 293-303.

Borchardt, M.A. (1996). Nutrients. *In* Stevenson RJ, Bothwell ML, Lowe RL (eds) *Algal Ecology. Freshwater Benthic Ecosystems*. Academic Press Inc, San Diego, CA, pp 183-227.

Carvalho, C.C.R., Fernandes P. (2010). Production of metabolites as bacterial responses to the marine environment. *Marine Drugs.8*, 705-727.

CDC-Centro de datos para la conservacion–Universidad Nacional Agraria La Molina. (1992). Estado de conservación de la diversidad natural de la región noroeste del Perú. La Molina.

CART-Comision ambiental regional de Tumbes, grupo técnico binacional. (2002). Plan Binacional para la gestión de recursos naturales fronterizos Tumbes (Perú), El Oro y Loja (Ecuador). Tumbes, Perú.

Chénier M.R., Beaumier D., Roy R., Driscoll B.T., Lawrence J.R., Greer C.W. (2003). Impact of seasonal variations and nutrient inputs on nitrogen cycling and degradation of

hexadecane by replicated river biofilms. *Applied Environmental Microbiology*. 69(9): 5170–5177.

Clarke, K.R., Warwick, R.M. (1994). *Change in marine communities: an approach to statistical analysis and interpretation*. Plymouth: Plymouth Marine Laboratory.

Droge, W. (2001). Free radicals in the physiological control of cell function. *Physiologycal Review,* 82: 47–95.

Farooq, A., Malfatti, F. (2007). Microbial structuring of marine ecosystems, *Nature Reviews Microbiology*. 5: 782-791.

Fenchel, T. (1993). There are more small than large species? *Oikos*, 68: 375-378.

Fenchel, T., Esteban, G.F., Finlay, B.J. (1997). Local versus global diversity of microorganisms: cryptic diversity of ciliated protozoa. *Oikos,* 80: 220–225

Fenchel, T., King, G.H., Blackburn, T.H. (1999). Bacterial biogeochemistry. Theecophysiology of mineral cycling. *International Microbiology*, 2: 201–204.

Field, J. G., Clarke, K.R., Warwick, R.M.. (1982). A pratical strategy for analysing multispecies distribution patterns. *Marine Ecology Progrese Series*, 8: 37–52.

Finlay, B.J., Maberly, S.C., Esteban, G.F. (1996). Spectacular abundance of ciliates in anoxic pond water: contribution of symbiont photosynthesis to host respiratory oxygen requirements. *FEMS Microbiology Ecology,* 20: 229-235.

Foyer, C.H., Bowshe C. (2004). Preface to plant carbon-nitrogen interactions from rhizosphere to planet. *Journal of Experimental Botany*, 55:4.

Giller, P.S., Hildrew, A.G., Raffaelli, D.G. (1994). *Aquatic ecology: Scale pattern and processes*. Blackwell, Oxford.

Harder, T., Lau, S.C.K., Dobretsov, S., Fang, T.K., Qian, P.Y. (2003). A distinctive epibiotic bacterial community on the soft coral *Dendronephthya* sp. and antibacterial activity of coral tissue extracts suggest a chemical mechanism against bacterial epibiosis. *FEMS Microbial Ecology*, 43: 337–347.

Holguin, G., Guzman, M.A., Bashan, Y. (1992). Two new nitrogen-fixing bacteria from the rhizosphere of mangrove trees: Their isolation, identification and *in vitro* interaction with rhizosphere *Staphylococcus* sp. *FEMS Microbiological. Ecology*, 101: 207-216.

Holguin, G., Vazquez, P., Bashan, Y. (2001). The role of sediment microorganisms in the productivity, conservation, and rehabilitation of mangrove ecosystems: an overview. *Biology and Fertility of Soils*, 33: 265-278.

Irani, K., Xia, Y., Zweier, J.L., Sollott, S.J., Der, C.J., Fearon, E.R., Sundaresan, M., Finkel, T., Goldschmidt-Clermont, P.J. (1997). Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts. *Science,* 275: 1649–1651.

Jones, D.P. (2006). Redefining oxidative stress. *Antioxid Redox Signal*, (9-10): 1865-1879.

Jones, D.P. (2008). Radical-free biology of oxidative stress. *American Journal of Cell Physiology,* 295: C849-C868.

Konstantinidis, K.T., Isaacs, N., Fett, J., Simpson, S., Long, D.T., Marsh, T.L. (2003). Microbial diversity and resistance to copper in metal-contaminated lake sediment. *Microbiological Ecology*, 45: 191–202.

Krawiec, Z., Bilinski, T., Schüller, C., Ruis, H. (2000). Reactive oxygen species as second messengers? Induction of the expression of yeast catalase T gene by heat and hyperosmotic stress does not require oxygen. *Acta Biochimica Polonia*, 47: 201-207.

Zdzisawa, K., Biliñski, T., Schüller, S., Ruis, H. (2000). Reactive oxygen species as second messengers? Induction of the expression of yeast catalase T gene by heat and hyperosmotic stress does not require oxygen. *Acta Bioquimica Polonia*, 47: 201–207.

Larsen, L.G., Harvey, J.W., Crimaldi, J.P. (2007). A delicate balance: Ecohydrological feedbacks governing landscape morphology in a lotic peatland. *Ecological Monographs*, 77: 591-614.

Li, Z., He, L., Wu, J., Jiang, Q. (2006). Bacterial community diversity associated with four marine sponges from the South China Sea based on 16S rRNA gene-DGGE fingerprinting. *Journal of Experimental Marine Biological Ecology*, 329: 75-85.

Liang, J.B., Chen, Y.Q., Chong, Y.L., Tam, N.F.Y., Zan, Q.J., Huang, L.N. (2007). Recovery of novel bacterial diversity from mangrove sediment. *Marine Biology*, 150: 739-747.

Magder, S. (2006). Reactive oxygen species: Toxic molecules or spark of life? *Critical Care*, 10: 208-215.

Muckian, L., Grant, R., Doyle, E., Clipson, N. (2007). Bacterial community structure in soils contaminated by polycyclic aromatic hydrocarbons. *Chemosphere*, 68: 1535-1541.

OMS (2003). *Manganese in drinking-water*. Documento de referencia para la elaboración de las Guías de la OMS para la calidad del agua potable. Ginebra (Suiza), Organización Mundial de la Salud (WHO/SDE/WSH/03.04/104).

Ortiz de Orué, D., Wedderhoff, I., Groves, M.R. (2012). ROS-mediated signalling in bacteria: Zinc-containing Cys-X-X-Cys redox centres and iron-based oxidative stress. *Journal of Signal Transduction*, 1: 1-9.

Peterson, C.G., Weibel, A.C., Grimm, N.B., Fisher, S.G. (1994). Mechanisms of benthic algal recovery following spates - comparison of simulated and natural events. *Oecologia*, 98: 280-290.

Qurashi, A.W., Sabri, A.N. (2011), Osmoadaptation and plant growth promotion by salt tolerant bacteria under salt stress. *African Journal of Microbiology Research*, 5: 3546-3554.

Raymond, J. (2005). The evolution of biological carbon and nitrogen cycling—a genomic perspective. *Reviews in Mineralogy & Geochemistry*, 59: 211-231.

Riemann, L., Steward, G. F., Azam, F. (2000). Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. *Applied Environmental Microbiology*, 66: 578–587.

Ritchie, L.E, Steiner J.M., Suchodolski, J.S. (2008). Assessment of microbial diversityalong the feline intestinal tract using16S rRNAgene analysis. *Microbiological Ecology*, 66: 590–598.

Sengupta, A., Chaudhuri, S. (1991). Ecology of heterotrophic dinitrogen fixation in the rhizosphere of mangrove plant community at the Ganges river estuary in India. *Oecologia*, 87: 560-564.

Suchodolski, J.S., Camacho, J., Steiner, J.M. (2008). Analysis of bacterial diversity in the canine duodenum, jejunum, ileum, and colon by comparative 16S rRNA gene analysis. FEMS *Microbiological Ecology*, 66: 487–498.

Taylor, N.L., Day, D.A., Millar, A.H. (2004). Targets of stress-induced oxidative damage in plant mitochondria and their impact on cell carbon/nitrogen metabolism. *Journal of Experimental Botany*, 55: 1–10.

Van der Grinten, E., Janssen, A.P.H.M., Barranguet, C., Admiraal, W. (2000). Microphytobenthos in temperate floodplain lakes: Experimental analysis of species distribution in environmental gradients. *Bulletin of Natural American Benthology Society*, 17: 117, 48th Annual Meeting NABS, Keystone, USA.

Van Dam, H., Mertens, A. (1995). Long-term changes of diatoms and chemistry in headwater streams polluted by atmospheric deposition of sulfur and nitrogen compounds. *Freshwater Biology*, 34: 579-600.

Van der Grinten, E., Janssen, M., Simis, S.G.H., Barranguet, C., Admiraal, W. (2004). Phosphate regime structuring species composition in cultured phototrophic biofilms. *Freshwater. Biology*, 49: 369-381.

Vazquez, P., Holguin, G., Puente, M.E., Lopez-Cortes, A., Bashan, Y. (2000). Phosphate-solubilizing microorganisms associated with the rhizosphere of mangroves in a semiarid coastal lagoon. *Biology and Fertility of Soils*, 30: 460-468.

Wetzel, R.G. (1983). *Limnology*. 2. Saunders College Publishing, Fort Worth: 753 pp.

Williams, P., Camara, M., Hardman, A., Swift, S., Milton, D., Hope, V.J., Winzer, K., Middleton, B., Pritchard, D.I., Bycroft, B.W. (2000). Quorum sensing and the population-dependent control of virulence. *Philos. Trans. R. Soc. London Series B Biological Sciences*, 355: 667–680.

- CAPÍTULO 3 -

Effects of copper to induce tolerant changes and recovery potential in composition of bacterial biofilms in mangrove ecosystems

Rafaela Elias Letts[1,2*], Silvia Sato[2], Diego Ugarte [2], Paola Yurivilca[2], and José M. Monserrat[1,3]

Manuscrip send to Microbial Ecology

[1]Programa de Pós-Graduação em Ciências Fisiológicas - Fisiologia Animal Comparada, Universidade Federal de Rio Grande – FURG, Rio Grande (RS), Brazil.

[2] Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Lima, Perú

[3]Instituto de Ciências Biológicas (ICB), Universidade Federal do Rio Grande - FURG

* Corresponding author

Av. Honório Delgado 430, San Martín de Porres. Lima-Peru

rafaela.elias@upch.pe, releltts@hotmail.com

Resumo

O objetivo deste estudo foi investigar biofilme tolerância comunidade poluição induzida (PICT), e recuperação de biofilme em cobre (Cu), a exposição, em dois diferentes comunidades bacterianas. As bactérias foram removidos do RT (altamente impactado pela poluição de metais) e sites de P (área de referência) de mangue no rio Tumbes, no Peru. Para avaliar o potencial induzido e recuperação nestas comunidades bacterianas, analisou-se as alterações da estrutura das comunidades bactérias expostas a 200 e 400 mg / L de Cu com uma pré-exposição a 3 mg / L deste metal e, em seguida, avaliados por electroforese em gel de gradiente desnaturante (DGGE). Um maior número de bactérias foram isoladas de campo e de laboratório comunidades de biofilme recolhidos e amostras de água. Após 3 dias de incubação em laboratório, os biofilmes de cobre tratados eram diferentes dos biofilmes de referência em bactérias composição e taxa de crescimento. Não houve diferenças nas proporções relativas de Gram-negativas e Gram-positiva entre o sítio P e RT foram registados, indicando a predominância de bactérias Gram-positivas (três vezes maior) em relação a bactérias Gram negativas. A indução moderado para PICT foi observada tolerância para as comunidades bacterianas em P local e uma resposta importante no site RT, com uma composição da comunidade em constante mudança.

Palavras-chave: Biofilme, cobre, poluição, mangue, recuperação.

Abstract

The aim of this study was to investigate biofilm pollution-induced community tolerance (PICT), and biofilm recovery under copper (Cu) exposure, in two different bacterial communities. Bacteria were removed from RT (highly impacted by metals pollution) and P (reference area) mangrove sites in Tumbes river, Peru. To evaluate induced and recovery potential in these bacterial communities, it was analyzed structure changes from bacteria communities exposed to 200 and 400 mg/L of Cu with a pre-exposure to 3 mg/L of this metal and then evaluated by denaturing gradient gel electrophoresis (DGGE). A larger number of bacteria were isolated from field and laboratory communities from collected biofilm and water samples. After 3 days of incubation in laboratory, copper treated biofilms were different from the reference biofilms in bacteria composition and growth ratio. No differences in the relative proportions of Gram-negative and Gram-positive between site P and RT were registered, showing a predominance of Gram-positive (three times higher) with respect to Gram negative. A moderated induction to tolerance PICT was observed for the bacterial communities in site P and a major response in RT site, with a constantly changing community composition.

Key words: Biofilm, copper, pollution, mangrove, recovery.

1. Introduction

In aquatic ecosystems, biofilms assume important key ecological functions such as primary production and nutrient cycling (Battin *et al*., 2003). These microbial assemblages are formed by phototrophic and heterotrophic communities characterized by short generation times. On the other hand, the fact that they are the first to interact with dissolved substances makes biofilms an interesting candidate for the early detection of the effects of chemicals on aquatic systems (Sabater *et al*., 2007). Bacterial biofilms commonly cover submersed surfaces in shallow freshwater systems. Especially in riverine systems, these biofilms have the capacity to modify the transport and accumulation of substances such as nutrients as well as organic toxicants and heavy metals (Barranguet *et al*, 2003). Biofilms have been frequently used to evaluate the effect of toxicants on benthic systems and also as indicators of water quality, since they give an integrated response of toxicants accumulation in the benthic environment (Genter and Lehmann, 2000, Flemming *et al*., 2007, Harrison *et al*., 2007). Bacterial communities are susceptible to anthropogenic disturbances like changes in oxygen concentrations and exposure to toxicants. For example, copper (Cu) has been shown to modify the structure and physiology of bacterial communities (Boivin *et al*., 2006). Copper exposure was also shown to change metabolism and induced the development of tolerance to copper in freshwater biofilms (Vymazal, 1984; Barranguet *et al*., 2002, 2003; Massieux *et al*., 2004; Lamberta *et al.*, 2012). This tolerance induced by pollution (PICT) are based in a chronic exposure to toxicant(s) that result in changes at the community level due to various toxicant-induced effects. The increase in Cu tolerance following a chronic exposure was previously described by Soldo and Behra (2000), Boivin *et al*. (2006) and Tlili *et al*. (2010). However, the capability of bacterial communities to recover (i.e., to return to the initial condition after toxicant exposure)

has rarely been investigated (Kelly and Tate, 1998; Griffiths *et al*., 2001; Kostov and Van Cleemput, 2001; Kiikkila *et al*., 2001; Lamberta *et al.*, 2012). Species diversity have sometimes been correlated to the capability of complex communities to recover after disturbance. Tilman and Dowing (1994), for instance, observed that the productivity of plant communities resisted disturbances when numerous plant species were present. Similar results were obtained for bacterial communities by Griffiths *et al*. (2000). They found that physiological recovery from chemical stress was more efficient at high bacterial diversity. Bacterial communities are generally very diverse and a high functional redundancy has been presumed so that communities may recover from a disturbance without losing any attribute (Finlay *et al*., 1997; Lamberta *et al.*, 2012). However, a functional recovery after a disturbance it does not necessarily mean that the community returned to its original species composition.

The main purpose of the present study was to test the ability of natural microbial biofilm communities to recover after copper exposure in laboratory mesocosm experiment. Given the structural and diversity complexity inherent to microbial biofilms, we hypothesized that recovery trajectories could vary between levels of heterogeneity of systems. Accordingly, a combination of methods was applied to assess microbial response after Cu exposure in terms of structure, diversity and growth.

2.Materials and methods

2.1.Analysis of water and mud

The characteristic of water and mud was determinated two times by year. The parameters tested were dissolved oxygen concentration, pH, temperature, conductivity and concentration of solids in suspension, all determined *in situ* in all sites analyzed, using a multi-parametric equipment (WTW). Nitrate, phosphates and potassium concentration was measured by means of a colorimetric kit (HANNA, model HI 83225 Grow Master). UVB intensity was determined by a Luxometer (Delta OHM) and the metal concentration in water and mud was analyzed by Optic-ICP.

2.2.Test site and sampling

The mangroves studied are located at the mouth of the Tumbes River, in Peru (03º25´LS and 80º17´LW) (Fig. 1). Two sites were studied: one site in Tumbes River (RT) extremely polluted by a former polymetalic mine and other at the Faculty fishing station (P) was considered a reference site. The two sites are influenced by UV radiation.

2.2.1 Gram Staining

Every UFC collected was stained with gram method (Gregersen, T., Rapid method for distinction of gram-negative from gram-positive bacteria, *Eur. J. Appl. Microbiol. Biotechnol.,* 5**,** 123, 1978) for this treatment we Add about 5 drops of crystal violet stain over the fixed culture. Let stand for 60 seconds. Pour off the stain and *gently* rinse the excess stain with a stream of water from a faucet or a plastic water bottle. Note that the objective of this step is to wash off the stain, not the fixed culture. Add about 5 drops of the iodine solution on the smear, enough to cover the fixed culture. Let stand for 30

seconds. Pour off the iodine solution and rinse the slide with running water. Shake off the excess water from the surface. Add a few drops of decolorizer so the solution trickles down the slide. Rinse it off with water after 5 seconds; Counterstain with 5 drops of the safranin solution for 20 seconds; Wash off the red safranin solution with water; Blot with bibulous paper to remove the excess water and air-dried.

Table 1. Physical and chemical variables (mean values and ± standard deviation (S:D)) during summer and winter seasons for two water bodies sampled in Tumbes mangrove: fishing station (P) and Tumbes River (RT) during period (2009-2011). The potassium and phosphate values correspond to baseline levels found in the water samples and the final values at the end of the trials.

	Tumbes river (RT)				Fishing station (P)			
Parameter	Humid		Dry		Humid		Dry	
	Mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.
pH	7.4	0.1	7.88	0.1	7.4	0.3	7.6	0.0
Dissolved oxygen (%)	18.2	3.3	20.3	1.2	13.0	1.2	17.0	0.2
Particles in suspension (ppm)	250.0	10.8	180.0	43.1	2,000.0	12.6	2,000.0	10.8
Temperature (°C)	26.2	0.2	25.4	1.1	27.6	0.9	26.0	1.7
Conductivity (µS)	127.0	2.3	105.0	55.6	3,999.0	0.0	3,999.0	0.0
UVB (W/m^2)	435.9	91.2	370.0	24.1	17.5	1.4	16.0	2.1
Potassium (mg/L) $_{initial}$	1.53	0.33	3.5	1.06	3.5	0.35	22.0	1.4
Phosphate mg/L) $_{initial}$	10.0	1.41	110.0	14.14	4.6	0.99	337.0	26.2
Potassium (mg/L) $_{final}$	0.5	0.35	1.0	0.21	1.0	0.35	5.0	1.4
Phosphate (mg/L) $_{final}$	5.4	0.71	78	1.41	2.5	0.35	300.0	14.1
Cu (mg/Kg)	986.4	65.3	1,790.4	108.3	214.4	11.6	214.4	28.3

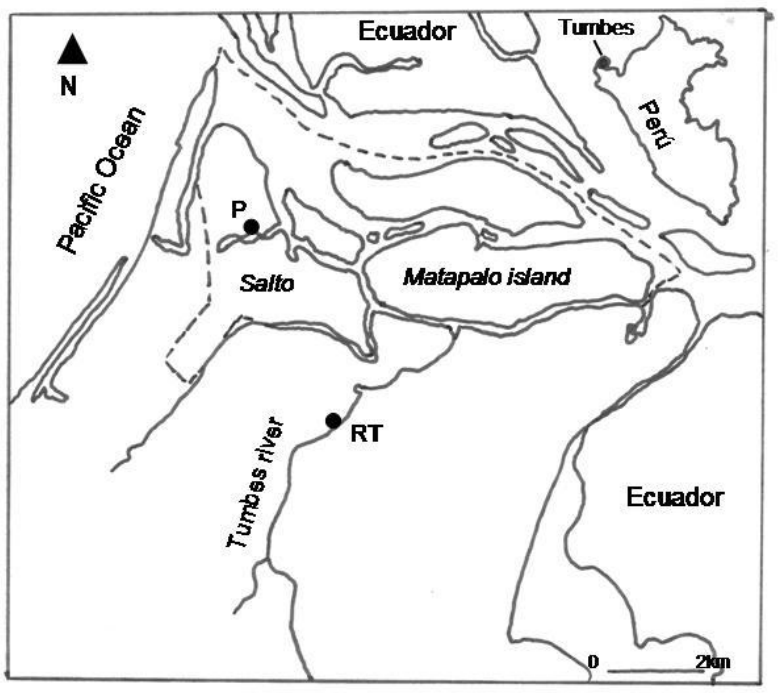


Figure 1. Location of the experimental sites in Tumbes mangrove: RT (Tumbes river; 17560527E 9605246N UTM) and P (Faculty station model 17567257E - 9612703N UTM) like low pollution site.

2.3.Experimental set-up

Two set of experiments were run: first, biofilms (1 mL) were collected on glass tubes during humid season in RT and P sites. Second, water (4 L) was sampled and biofilms were grown on glass discs (1.5 cm^2 surface) with water from RT or P sites. The glass tubes and discs were used for induction of tolerance experiments.

In tolerance to copper exposure it was assayed a Cu nominal concentration of 3 ppm of Cu that we calling pre-exposure and in parallel it was run a control group without Cu to follow bacteria associated with biofilm growth during three weeks. Them we determined total carbohydrate like oligosaccharides and polysaccharides by (Dubois *et*

al en 1956) methods to quantify a concentration of biofilm to start the exposure; we did not quantify the end of exposure. Biofilms samples were grown on glass discs with Tryptona Soya Agar (TSA) suplemented with 200 or 400 ppm of nominal Cu during four days. For biofilms generated from water samples, it was taken the entire biofilm formed on the glass discs to grown in TSA suplemented with 200 or 400 ppm of nominal Cu. To evaluate the response of biofilm exposed to high concentration 400 ppm (designated as the highest based on annual reports), we grown biofilms taken from 200 ppm to 400 ppm and biofilms grown in 400 ppm to 200 ppm of nominal Cu. Finally it was exposed biofilms to water without copper for four weeks in order to evaluate recuperation. After every period of four weeks it was quantified units of forming colonies (CFU) generated and taken samples for PCR analysis.

2.4.PCR and denaturing gradient gel electrophoresis (DGGE)

The genetic structure of the bacterial communities of every treatment and biofilm samples was characterized using DGGE. Bacterial specific polymerase chain reaction (PCR)-DGGE was performed according to the method described by Muyzer *et al*. (1993) and further adapted by Massieux *et al.* (2004). Bacterial specific primers were used for 16S rRNA gene amplification by PCR of extracted DNA obtaining a 450-bp amplicon. Universal bacterial primers (final concentration: 0.25 µM for each primer) were employed: F-341 (5'-CCTACGGGAGGCAGCAG-3') and R-786 (5'-GACTACCAGGGTATCTAATC-3') (Ritchie *et al*., 2008). Reactions were performed with the kit HotStart HiFidelity (Qiagen), using 60 ng of DNA template. To screen for potential contamination of PCR reagents, a negative PCR control using H_2O instead of a

DNA template was used. The samples were amplified in a thermocycler (Mastercycler Gradient, Eppendorf AG, Hamburg, Germany), using the following PCR protocol: initial denaturing at 95 ºC for 3 min, 30 cycles of denaturation at 95 ºC for 30 s, annealing at 54 ºC for 30 s, extension at 72 ºC for 1 min, and a final extension at 72 ºC for 10 min (Muyzer *et al*., 1993). The DGGE with linear gradient of urea and formamide between 30% and 60% (100% denaturing conditions are defined as 7 M urea and 40% [v/v] formamide), ranged from 35% at the top to 60% at the bottom of the gel. The gel was submerged in a Tris acetate EDTA buffer (0.5X TAE) containing 40 mM Tris, 40 mM acetic acid, 1 mM EDTA, pH 7.6 and submitted to 75 V for 16 h. The parameters that were included in the analysis of bands were: number of bands, molecular size and intensity of the bands.

2.5.Determination of bacterial induced tolerance

The induction of tolerance to copper was considered positive if the CFU significantly increased its growth when incubated with an increased copper concentration.

The biofilms of bacteria communities suspensions were diluted in saline solution (0.85%) in order to remove the extra polymeric matrix by sonication for 10 min and 40 Hz (Branson 8510). Then the suspensions were centrifuged for 5 min to 1129.87 xg and the bacteria pellets were kept. Samples were serially diluted three times in a dilution of 1:100 in saline solution and plating. These diluted bacterial suspensions were exposed to $CuCl_2$ (3 ppm, 200 ppm and 400 ppm) nominal concentrations, the culture was maintained at 25 ºC and bacterial growth was monitored during 72 hours, being counted the number of CFU formed.

2.6.Statistical analysis

The shifts between bacterial communities determined with DGGE profiling were graphically represented in a non-metric multidimensional scaling (MDS), which represents the matching similarities calculated in a triangular matrix of similarity coefficients computed between every pair of samples (Warwick and Clarke, 1998). It was employed the Bray –Curtis measure of similarity (Bray and Curtis, 1957) between every pair of studies. A one-way ANOVA was used to statistically compare the growth ratio between copper concentrations, employing Bonferroni method as post-hoc test. Previously normality and variance homogeneity assumptions were verified. In all cases, a significance level of 5% was employed.

3. Results

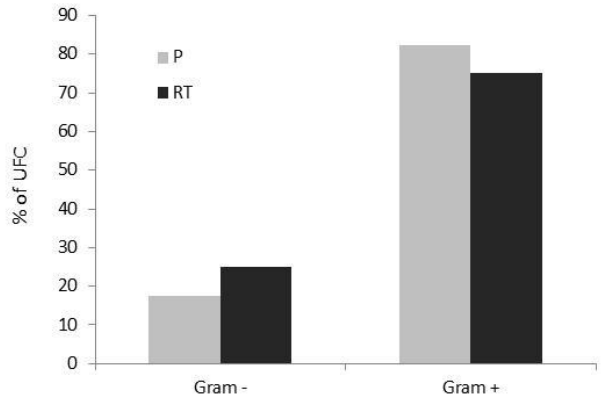


Figure 1. Percentage of Gram- and Gram + bacteria found in biofilms communities sampled at RT and P sites.

CFU that grown in TSA medium showed a prevalence of Gram+ for both P and RT, with statistical significance ($p<0.0001$), (Fig. 1). This difference was maintained during the different phases assayed (pre-exposure, exposure and recovery).

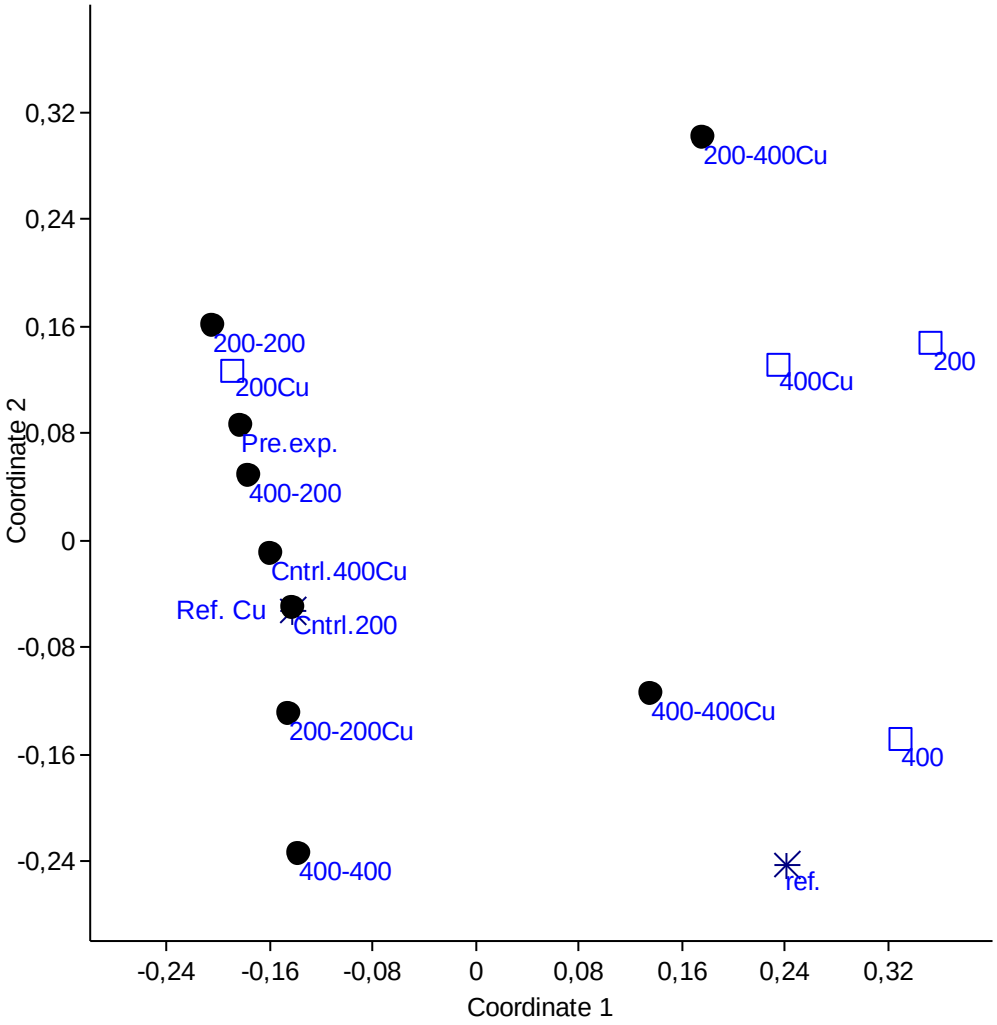


Figure 2. Non-metric multidimensional scaling (MDS) of denaturing gel electrophoresis (SDS-PAGE) of biofilm samples from different aquaria. Asterisks: freshly collected biofilm. Black points: samples exposed to 3 ppm, 200 ppm or 400 ppm

of copper. The "-" indicates transference concentration treatment between 200 and 400 ppm. White squares: samples after recovery treatment (0 ppm of Cu) for site P.

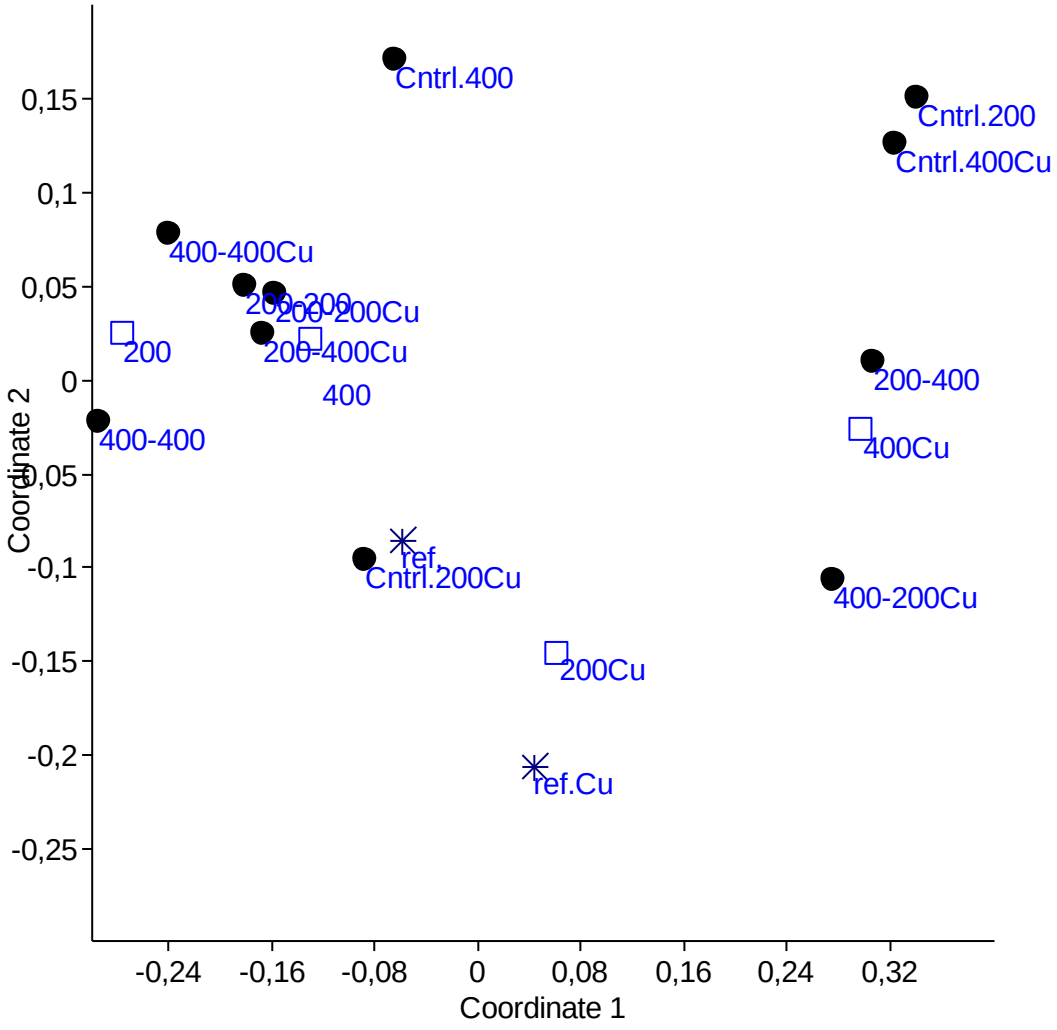
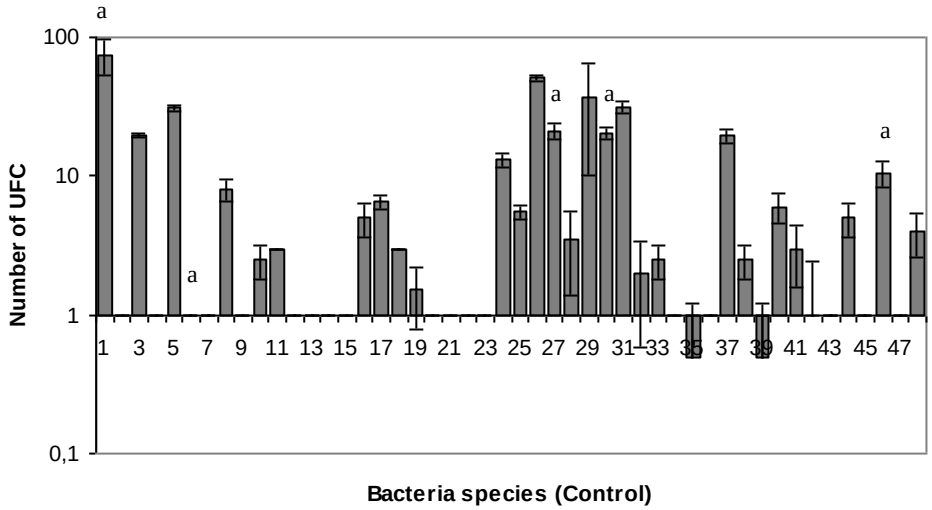


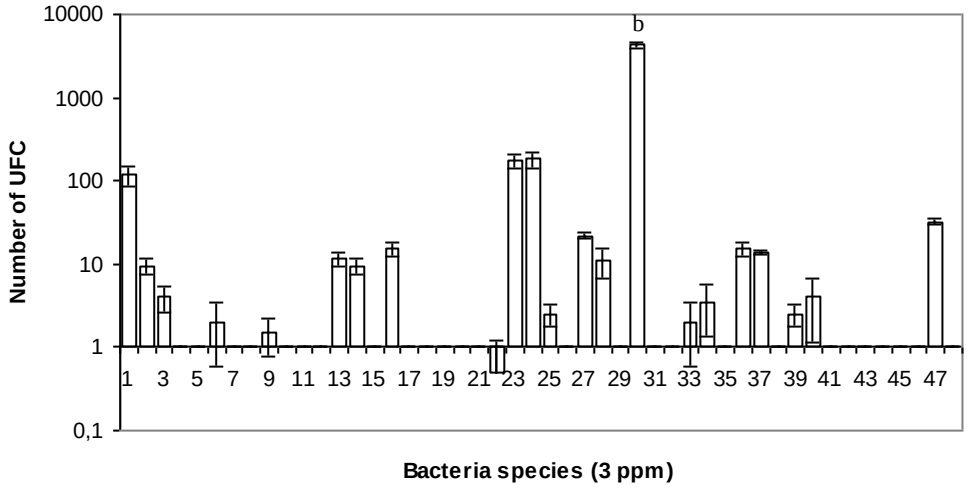
Figure 3. Non-metric multidimensional scaling (MDS) of denaturing gel electrophoresis (SDS-PAGE) of biofilm samples from different aquaria. Asterisks: freshly collected biofilm. Black points: samples exposed to 3 ppm, 200 ppm or 400 ppm

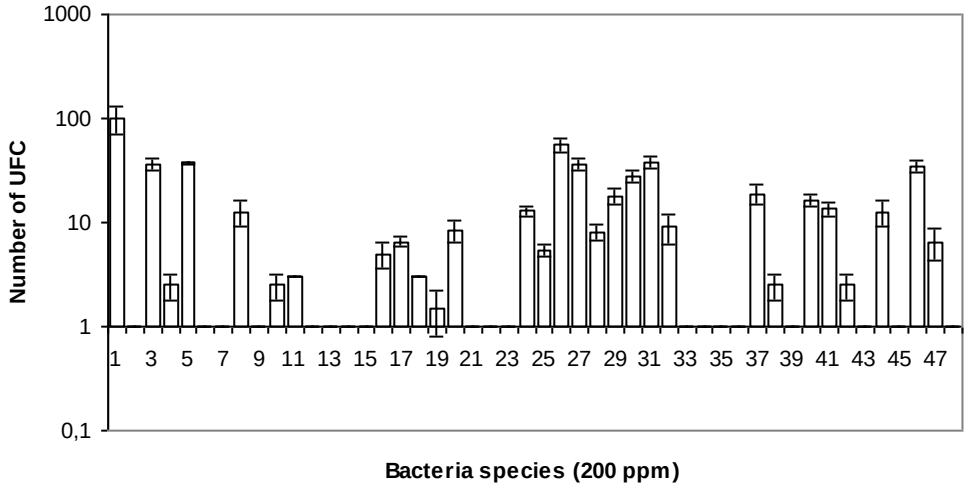
of copper. The "-" indicates transference concentration treatment between 200 and 400 ppm. White squares: samples after recovery treatment (0 ppm of Cu) for site RT.

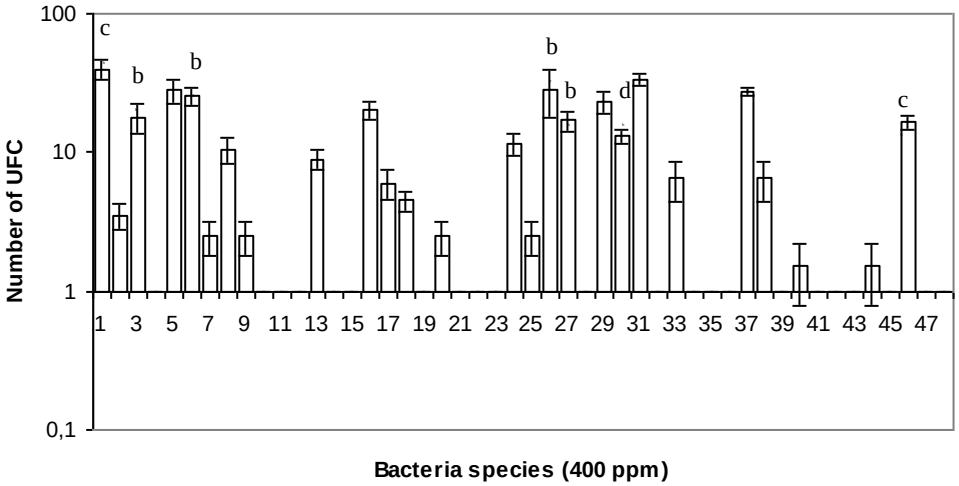
Based on MDS test, the result showed increased distance between different events (pre exposure, exposure and recovery). Figure 2 shows the MDS corresponding to the P site, where there is a grouping of copper treatments, including the reference sample. During the recovery period, the distance increased with respect to control and reference samples from both sites. Assuming that the differences between aquaria are primarily caused by the different exposure concentrations of copper, it seems that copper had a significant effect on the distance relation and diversity changes on bacterial community (Fig 3).

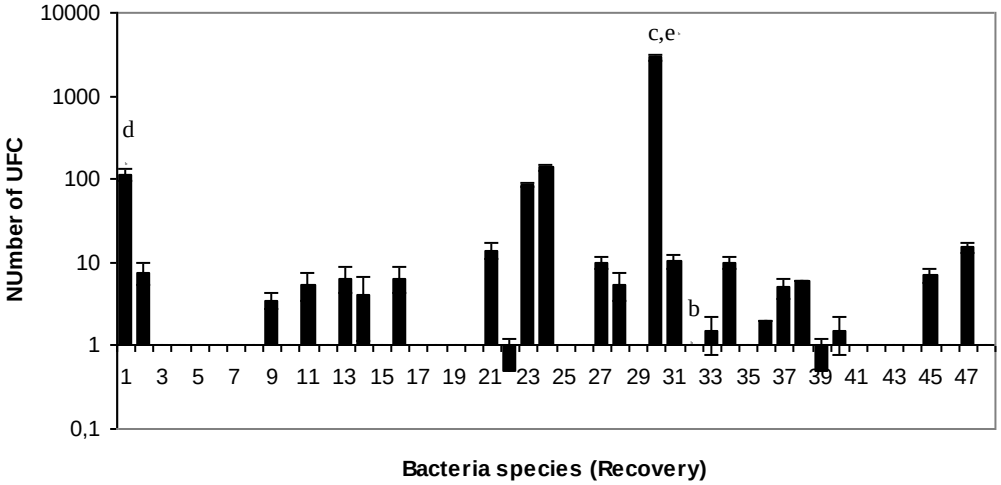
4a.


a
a
a
a
100
10
1
0,1
Number of UFC
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47
Bacteria species (Control)

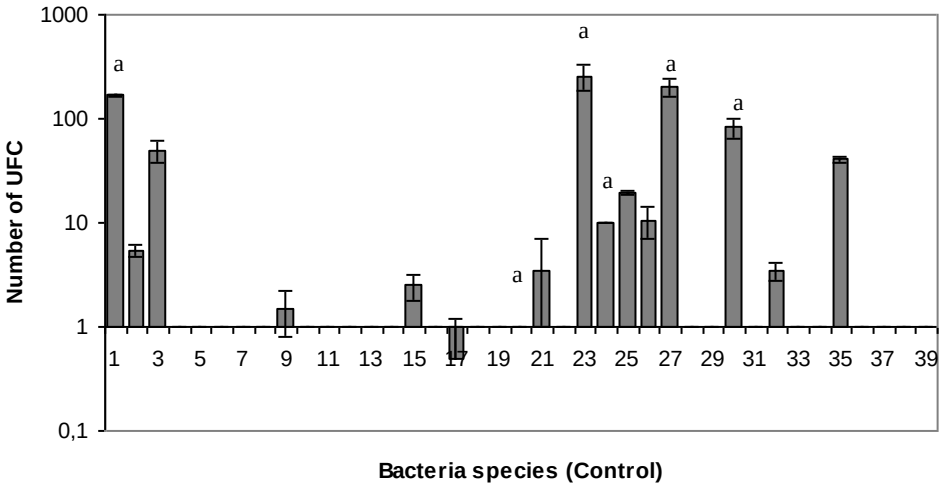

b
10000
1000
100
10
1
0,1
Number of UFC
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47
Bacteria species (3 ppm)

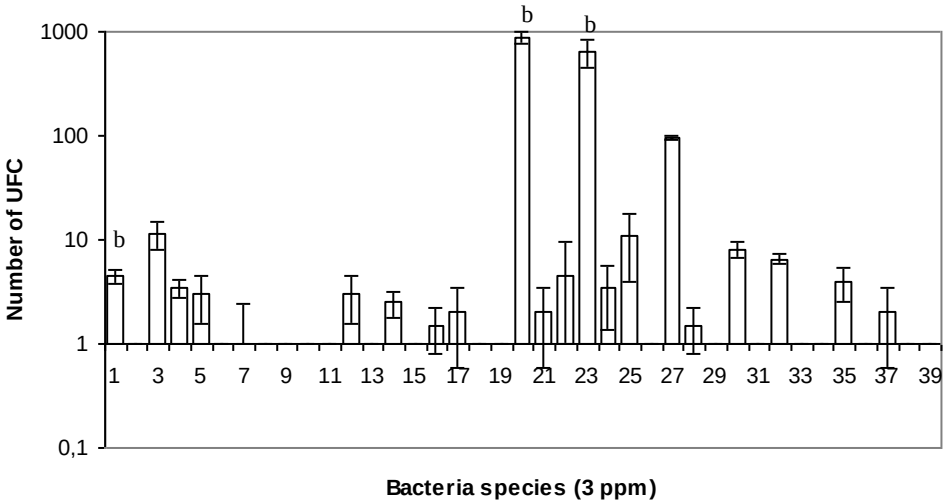

1000
100
10
1
0,1
Number of UFC
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47
Bacteria species (200 ppm)

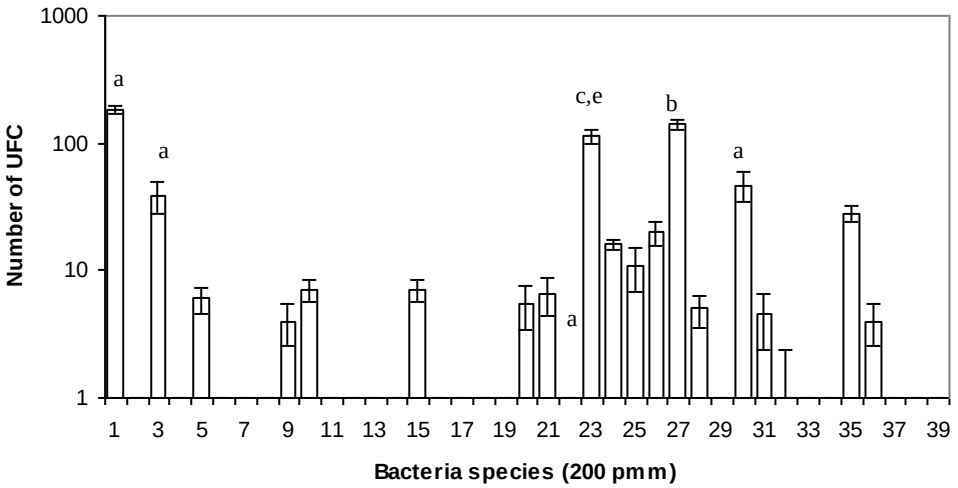

100
10
1
0,1
Number of UFC
c
b
b
b
b
d
c
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47
Bacteria species (400 ppm)


10000
1000
100
10
1
0,1
NUmber of UFC
d
c,e
b
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47
Bacteria species (Recovery)

4b.


1000
100
10
1
0,1
Number of UFC
a
a
a
a
a
a
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39
Bacteria species (Control)


1000
100
10
1
0,1
Number of UFC
b
b
b
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39
Bacteria species (3 ppm)


1000
100
10
1
Number of UFC
a
a
a
c,e
b
a
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39
Bacteria species (200 pmm)

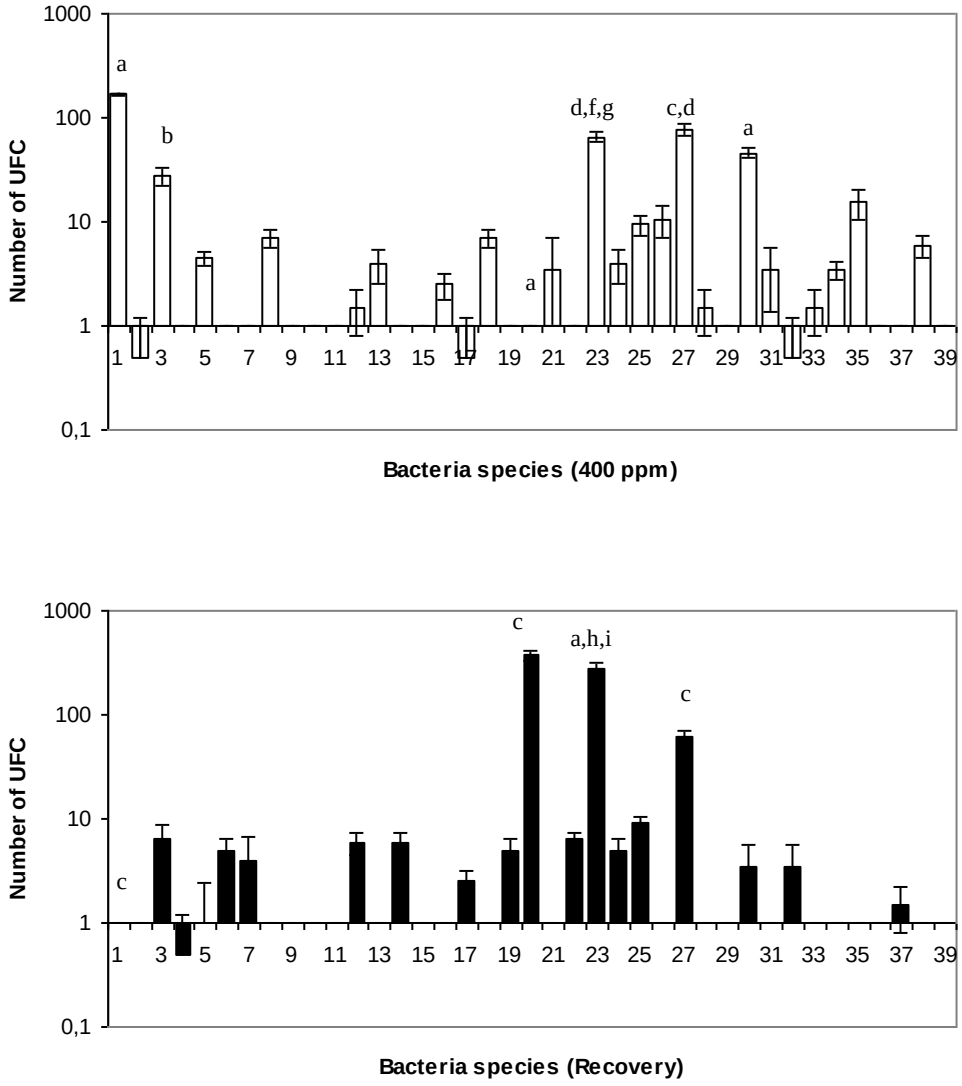


Figure 4a,b. The median of the UFC generated of aquatic bacterial communities from biofilms exposed to different copper concentrations; 0 ppm Cu: grey bars, 3 ppm Cu (Pre Exposure), 200 ppm Cu, 400 ppm Cu white bars and recovery treatment 0 ppm Cu black bars. The error bars represent 95% confidence intervals. a. site P and b. site RT.

The numbers of CFU showed significant differences under the different experimental conditions of bacteria communities exposed to 0, 3, 200, or 400 ppm of copper and the recovery period (Fig. 4). However, the sub-samples of the bacterial communities exposed to 400 ppm of copper had a lower CFU number with respect to

control. Furthermore the recovery period the number of CFUs was kept below certain levels in the control CFUs. We observe the increased number of CFUs from 3 ppm of copper treatment respect to control in both sites P and RT. Those differences in bacterial growth were maintained until the end of the experiment (Fig. 4).

Tolerance characteristics for copper were determined using growth ratio of CFUs. In Fig. 4, the copper-tolerance of the different bacterial communities is shown. The bacterial communities from the reference aquarium showed variable copper-tolerance in time by increased growth ratio in P and RT. Growth being CFUs always increasing with different copper concentrantions except with 400 ppm. All different period showed significant differences under the given experimental conditions between bacterial communities exposed to 0, 3, 200 and 400 ppm of copper and recovery (Fig. 4).

4.Discussion

Copper will affect each biofilm group to a different degree and lead to modifications not only of each component but also in the role that such component may play in the trophic web (Barranguet *et al.*, 2003). In the present study the potential of bacterial communities associated in freshwater biofilms to recover after copper exposure was investigated. DGGE and growth ratio shifts in bacterial communities were shown to correlate with copper exposure concentrations in the aquaria. The observation is consistent with the conclusions of Finlay *et al*. (1997) on the diversity of bacterial taxa, where it was demonstrated a high level of redundancy in bacterial communities that allowed them in the adaptation to new conditions. Under this view, it seems that the copper-induced successional changes during the exposure period remained present in

the recovery period, despite bacterial re-inoculation via water refreshments using natural surface water. It could be expected that bacterial communities from biofilms exposed to the different copper concentration would regain their uniform genetic structure more quickly when bacterial communities are re-inoculated to the system during the recovery period via natural surface water. Several explanations may be brought forward explaining why the genetic differences were maintained as showed in Fig. 2 and Fig. 3. Because a close relationship is expected between autotrophic and heterotrophic microorganisms (Romani *et al*., 2004), persistent differences of bacterial community composition could theoretically be maintained via long lasting effects of copper on the autotrophic microorganisms that dominated the biofilm (Massieux *et al*., 2004). Although several numbers of studies on the impact of toxicants on biofilm microbial communities have been performed, knowledge on their recovery dynamics following a decrease in exposure levels remains scarce (Boivin *et al*., 2006; Dorigo *et al*., 2010; Rotter *et al*., 2011). The growth profiles of bacterial communities were significantly different upon copper exposure, but these differences were lost after recovery (Fig. 4). Griffiths *et al*. (2000) observed functional recovery of bacterial communities after a heat shock, while the structure of the bacterial communities, based on DGGE analysis remained different. Two months after a heat shock, all treated and untreated bacterial communities employed grass residues to the same extent. However, in that study, bacterial communities were unable to utilize grass residue to the same extent after a persistent copper stress, pointing that the resilience observed after heat stress in fact did not occurred with other stressful factor as copper pollution. Kiikkila *et al*. (2001) made similar conclusions after studying copper-exposed microbial communities using phospholipids fatty acid analysis (PLFA) finding an bacteria change composition after Cu treatment. The present experiment did not show a complete loss of

tolerance after the recovery period, particularly with samples from P site. A but a plausible reason for this may be the environment origin, since in the case of the P site, the constant salinity variations which are subject the bacterial communities of this estuarine region impose a greater degree of tolerance to pollution (Singer *et al*., 2010). Genetically different communities can have similar tolerance for a contaminant for the same reasons as these may be capable to growth with more efficiency. Therefore, in view of our results, it seems that the exposure to a contaminant determined the level of tolerance to that contaminant and not the community composition or structure that shift permanently.

Acknowledgments

The research was supported by a Doctoral fellowship from FINCyT-LASPAU agency Authors wish to thank P. Yurivilca for his invaluable logistic support. We would like to thank Tumbes University and Jorge Echevarria and INRENA for kindly supplying information from the studied sites. J.M. Monserrat receives a productivity research fellowship from the Brazilian agency CNPq (process number PQ 306027/2009-7)

References

Barranguet, C., Plans, M., Van der Grinten, E., Sinke, J.J., Admiraal, W. (2002). Development of photosynthetic biofilms affected by dissolved and sorbed copper in a eutrophic river. *Environmental Toxicology and Chemistry* 21, 1955-1965.

Barranguet, C., Van den Ende, F.P., Rutgers, M., Breure, A.M., Greijdanus, M., Sinke, J.J., Admiraal, W. (2003). Copper-induced modifications of the trophic relations in riverine algal-bacterial biofilms. *Environmental Toxicology and Chemistry* 22, 1340-1349.

Battin, T.J., Kaplan, L.A., Newbold, J.D., Hansen, C.M.E., (2003). Contributions of microbial biofilms to ecosystem processes in stream mesocosms. *Nature* 426, 439-441.

Boivin M.Y., Massieux B., Breure A.M., Greve G.D. Rutgers M. and Admiraal W. (2006). Functional recovery of biofilm bacterial communities after copper exposure. *Environmental Pollution* 140, 239-246.

Bray, J.R. and Curtis, J.T. (1975). An ordination of the upland forest communities of Southern Wisconsin. *Ecological Monographs* 27,325-349.

Dorigo, U., Bérard, A., Rimet, F., Bouchez, A., Montuelle, B., (2010). *In situ* assessment of periphyton recovery in a river contaminated by pesticides. *Aquatic Toxicology* 98, 396-406.

Finlay, B.J., Maberly, C., Cooper, J.I. (1997). Microbial diversity and ecosystem function. *Oikos* 80, 209-213.

Flemming H., Neu T.R., and Wozniak D.J. (2007). The EPS Matrix: The "house of biofilm cells". *Journal of Bacteriology* 189, 7945–7947.

Genter R.B, Lehmann R.M. (2000). Metal toxicity inferred from algal population density, heterotrophic substrate use, and fatty acid profile in a small stream. *Environmental Toxicology and Chemistry* 19, 869-878.

Griffiths, B.S., Ritz, K., Bardgett, R.D., Cook, R., Christensen, S., Ekelund, F., Sorensen, S.J., Baath, E., Bloem, J., de Ruiter, P.C., Dolfing, J., Nicolardot, B. (2000). Ecosystem response of pasture soil communities to fumigation-induced microbial diversity reductions: an examination of the biodiversity-ecosystem function relationship. *Oikos* 90, 279-294.

Griffiths, B.S., Ritz, K., Wheatley, R., Kuan, H.L., Boag, B., Christensen, S., Ekelund, F., Sorensen, S.J., Muller, S., Bloem, J. (2001). An examination of the biodiversity-ecosystem function relationship in arable soil microbial communities. *Soil Biology and Biochemistry* 33, 1713-1722.

Harrison J.J., Ceri H. and Turner R.J. (2007). Multimetal resistance and tolerance in microbial biofilms. *Nature Reviews Microbiology* 5, 928-938

Kelly, J.J., Tate, R.L. (1998). Effects of heavy metal contamination and remediation on soil microbial communities in the vicinity of a zinc smelter. *Journal of Environmental Quality* 27, 609-617.

Kiikkila, O., Perkiomaki, J., Barnette, M., Derome, J., Pennanen, T., Tulisalo, E., Fritze, H. (2001). In situ bioremediation through mulching of soil polluted by a copper-nickel smelter. *Journal of Environmental Quality* 30, 1134-1143.

Lamberta A., Morinb S, Artigasa J., Volata B., Coquerya M., Neyraa M., Pescea S. (2012). Structural and functional recovery of microbial biofilms after a decrease in copper exposure: Influence of the presence of pristine communities. *Aquatic Toxicology* 109, 118-126.

Gregersen, T., Rapid method for distinction of gram-negative from gram-positive bacteria, *Eur. J. Appl. Microbiol. Biotechnol.*, 5**,** 123, 1978

Kostov, O., Van Cleemput, O. (2001). Microbial activity of Cu contaminated soils and effect of lime and compost on soil resiliency. *Compost Science and Utilization* 9, 336-351.

Massieux, B., Boivin, M.E.Y., Van den Ende, F.P., Langenskiöld, J.,Marvan, P., Barranguet, C., Admiraal, W., Laanbroek, H.J.,Zwart, G. (2004). Analysing structural and physiological profiles to assess the effect of Cu on biofilm forming microbial communities. *Applied Environmental Microbiology* 70, 4512-4521.

Muyzer, G., Dewaal, E.C., Uitterlinden, A.G. (1993). Profiling of complex microbial-populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S ribosomal-RNA. *Applied Environmental Microbiology* 59, 695-700.

Outten, F.W., Huffman, D.L., Hale, J.A. & O'Halloran, T.V. (2001). The independent cue and cus systems confer copper tolerance during aerobic and anaerobic growth in Escherichia coli. *J. Biol. Chem.* 276, pp. 30670-30677.

Ritchie, L.E, Steiner J.M., and Suchodolski, J.S. (2008). Assessment of microbial diversityalong the feline intestinal tract using16S rRNAgene analysis. *Microbiology Ecology* 66, 590–598.

Romani, A.M., Guasch, H., Munoz, I., Ruana, J., Vilalta, E., Schwartz, T., Emtiazi, F., Sabater, S. (2004). Biofilm structure and function and possible implications for riverine DOC dynamics. *Microbial Ecology* 47, 316-328.

Rotter, S., Sans-Piché, F., Streck, G., Altenburger, R., Schmitt-Jansen, M. (2011). Active biomonitoring of contamination in aquatic systems – An *in situ* translocation experiment applying the PICT concept. *Aquatic Toxicology* 101, 228-236.

Sabater, S., Guasch, H., Ricart, M., Romani, A., Vidal, G., Klünder, C., Schmitt-Jansen, M., (2007). Monitoring the effect of chemicals on biological communities. The biofilm as an interface. *Analytical and Bioanalytical Chemistry*, 387, 1425-1434.

Satish Kumar Singh (2009).. 2009). Mechanisms of bacterial copper detoxification and oxygen reuction in cueo and chemotactic signal amplification by receptor clustering . The Uninversity of Arizona..

Singer G, Besemer K, Schmitt-Kopplin P, Hödl I, Battin T.J. (2010). Physical heterogeneity increases biofilm resource use and its molecular diversity in stream mesocosms. *PLoS ONE* 5, 9988-9999.

Soldo, D., Behra, R. (2000). Long-term effects of copper on the structure of freshwater periphyton communities and their tolerance to copper, zinc, nickel and silver. *Aquatic Toxicology* 47, 181-189.

Tilman, D., Dowing, J.A. (1994). Biodiversity and stability in grasslands. *Nature* 367, 363-365.

Tlili, A., Bérard, A., Roulier, J.L., Volat, B., Montuelle, B. (2010). PO4 3- dependence of the tolerance of autotrophic and heterotrophic biofilm communities to copper and diuron. *Aquatic Toxicology* 98, 165-177.

Vymazal, J. (1984). Short-term uptake of heavy metals by periphyton algae. *Hydrobiologia* 119, 171-179.

Warwick R.M., Clarke K.R. (1998). Taxonomic distinctness and environmental assessment. *Journal of Applied Ecology* 35, 532-543.

CONCLUSÕES GERAIS

Com base nos resultados obtidos nos diferentes capítulos apresentados, pode-se concluir que:

1- O processo de sucessão das comunidades bacterianas amostradas em dois locais mostraram uma resposta associada com a presença de contaminação de cobre. (Capitulo 1).

2- Os resultados obtidos mostraram evidências de que a temperatura e os nutrientes são fatores importantes que regulam a estrutura dos biofilmes, sendo estas respostas ainda afetadas por metais como o cobre. A utilização do mesocosmos avaliou o desenvolvimento de comunidades bactérianas a partir de amostras coletadas em dois sítios com diferenças em termos de nutrientes, salinidade e concentração de metais (Capitulo 2).

3- A composição de espécies bacterianas depois dos tratamentos de troca de temperatura, nutrientes e cobre nominal foram diversificando-se muito lentamente, dependendo do local onde as amostras foram coletadas. Assim, a utilização de mesocosmos se apresentou como uma alternativa viável para analisar a composição de espécies em relação aos processos altamente dinâmicos de crescimento e perturbação (Capitulo 2).

4- Não foi observada uma perda completa de tolerância ao cobre após o período de recuperação da exposição a fatores ambientais e de cobre, mais a queda foi maios no sítio P em relação ao parâmetro de crescimento. Uma explicação plausível para isto pode ser a origem do ambiente onde as amostras foram coletadas, já que no sitio P, que apresenta importantes variações de salinidade, foi observado que as comunidades bacterianas apresentaram um maior grau de tolerância à poluição (Capitulo 3).

5- Comunidades com composição e estrutura diferentes podem ter níveis semelhantes de tolerância contra um único poluente. Como os resultados mostram, a tolerância para um determinado poluente está relacionada com a capacidade de alterar permanentemente a estrutura e a composição da comunidade bacteriana (Capitulo 3).

Finalmente, e como conclusão geral, pode-se afirmar que a resistência intrínseca (resiliência) em alguns biofilmes a fatores externos perturbadores está fortemente relacionada com fatores ambientais e seu grau de sucessão e desenvolvimento. Sugere-se que a avaliação das implicações dessas mudanças na diversidade de bactérias sobre outras comunidades que compartilham e conformam o biofilme como diatomáceas e algas, sendo a avaliação da capacidade funcional fisiológica desses sistemas e seu potencial impacto sobre o ecossistema é uma possível continuação deste trabalho.

REFERÊNCIAS BIBLIOGRÁFICAS DA INTRODUÇÃO

Battin T.J., W.T. Sloan, S. Kjelleberg, H. Daims, I.M. Head, T.P. Curtis, L. Eberl (2007). Microbial landscapes: New paths to biofilm research. *Natural Review Microbiology* 5: 76–81.

Biggs R., S.R. Carpenter, W.A. Brock (2009). Turning back from the brink: Detecting an impending regime shift in time to avert it. *Proceedings of the National Academy of Sciences* 106: 826-831.

Blanck H., B. Dahl (1996). Pollution-induced community tolerance (PICT) in marine periphyton in a gradient of tri-n-butyltin (TBT) contamination. *Aquatic Toxicology* 35: 59-77.

Blanck H., S-Å. Wängberg (1988a). Validity of an ecotoxicological test system: Short-term and long-term effects of arsenate on marine periphyton communities in laboratory systems. *Canadian Journal of Fish Aquatic Sciences* 45: 1807-1815.

Blanck H., S-Å. Wängberg (1988b). Induced community tolerance in marine periphyton established under arsenate stress. *Canadian Journal of Fish Aquatic Sciences* 45: 1816-1819.

Blanck H., S-Å. Wängberg, S. Molander (1988). Pollution-induced community tolerance - A new ecotoxicological tool. Em: Cairns J. Jr. e J.R. Pratt (eds) *Functional*

Testing of Aquatic Biota for Estimating Hazards of Chemicals. ASTM STP 988. American Society for Testing of Materials, Philadelphia, PA, pp. 219-230.

Blum J.L. (1957). An ecological study of the algae of the Saline River, Michigan. *Hydrobiologia* 9: 361-408.

Buettner, G.R. & Jurkiewicz, B.A. (1996). Catalytic metals, ascorbate and free radicals: Combinations to avoid. *Radiat. Res.* 145, pp. 532-541.

Carpenter S.R., W.A. Brock. (2006). Raising variance: A leading indicator of ecological transition. *Ecology Letters* 9: 311-318.

Chase J.M. (1999). Food web effects of prey size refugia variable interactions and alternative stable equilibria. *American Naturalist* 154: 559-570.

Chase J.M. (2003). Experimental evidence for alternative stable equilibria in a benthic pond food web. *Ecology Letters* 6: 733-741.

Chaves L.S., Simões M. and Vieira M.João. (2012). A comparative study of drinking water biofilm monitoring with flow cell and Propella™ bioreactors. *Water Science & Technology: Water Supply* 3: 334–342.

Connell J.H. (1978). Diversity in tropical rain forests and coral reefs. *Science*, 199: 1302-1310.

Dakos V., M. Scheffer, E.H. Van Nes, V. Brovkin, V. Petoukhov, H. Held (2008). Slowing down as an early warning signal for abrupt climate change. *Proceedings of the National Academy of Sciences* 105: 14308-14312.

Dakos V., E.H. Van Nes, R. Donangelo, H. Fort, M. Scheffer (2010). Spatial correlation as leading indicator of catastrophic shifts. *Theoretical Ecology* 3: 163-174.

Deniseger J., A. Austin, W.P. Lucey (1986) Periphyton communities in a pristine mountain stream above and below heavy metal mining operations. *Freshwater Biology* 16: 209-218.

Demoling A.L., Baath E. (2008). No long-term persistance of bacterial pollution-induced community tolerance in tylosin polluted soil. *Environmental Science & Technology*, 118: 6917-6921.

EEA (1994). European Rivers and Lakes. Assessment of their Environmental State. EEA Environmental Monographs 1. Kristensen P and Hansen HO (eds), European Environmental Agency, Copenhagen, Denmark.

EEA (2012). European bathing water quality in 2011. EEA Report No 3. European Environmental Agency, Copenhagen, Denmark.

Fechner Lise C., Versace Francois, Gourlay-France C., Tusseau-Vuillemin M., H. (2011). Adaptation of copper community tolerance levels after biofilm transplantation in an urban river. *Aquatic Toxicology* 106: 32-41.

Franke, S., Grass, G., Rensing, C. & Nies, D.H. (2003). Molecular analysis of the copper-transporting efflux system CusCFBA of Escherichia coli. *J. Bacteriol.* 185, pp. 3804-3812.

Forman R.T.T., M. Godron (1981). Patches and structural components for a landscape ecology. *Bioscience* 31:733-740.

Foster P.L. (1982). Metal resistances of chlorophyta from rivers polluted by heavy metals. *Freshwater Biology* 12: 41-61.

Gray B.R., W.R. Hill (1995). Nickel sorption by periphyton exposed to different light intensities. *Journal of the North American Benthological Society* 14: 299-305.

Grass, G. & Rensing, C. (2001). Genes involved in copper homeostasis in Escherichia coli. *J. Bacteriol.* 183, pp. 2145-2147.

Genter R.B. (1996). Ecotoxicology of inorganic chemical stress to algae. VII. Response of algal communities to inorganic stressors. Em: Stevenson R.J., M.L. Bothwell, R.L. Lowe (eds) *Algal Ecology. Freshwater Benthic Ecosystems*. Academic Press, San Diego, CA, pp. 452-457.

Genter R.B., D.S. Cherry, E.P. Smith, J.Jr. Cairns (1987). Algal-periphyton population and community changes from zinc stress in stream mesocosms. *Hydrobiologia* 153: 261-275.

Green D.G., S. Sadedin. (2005). Interactions matter - complexity in landscapes and ecosystems. *Ecological Complexity* 2:117-130.

Gustafson E.J. (1998). Quantifying landscape spatial pattern: What is the state of the art? *Ecosystems* 1: 143-156.

Gustavson K., S-Å. Wängberg (1995). Tolerance induction and succession in microalgae communities exposed to copper and atrazine. *Aquatic Toxicology* 32: 283-302.

Guttal V., C. Jayaprakash. (2009). Spatial variance and spatial skewness: leading indicators of regime shifts in spatial ecological systems. *Theoretical Ecology* 2: 3-12.

Harrison G.W. (1986). Multiple stable equilibria in a predator-prey system. *Bulletin of Mathematical Biology* 48: 137-148.

Herman P.M.J., J.J. Middelburg, J. Widdows, C.H. Lucas, C.H.R. Heip. (2000). Stable isotopes as trophic tracers: Combining field sampling and manipulative labelling of food resources for macrobenthos. *Marine Ecology Progress Series* 204: 79-92.

Hutchinson G.E. (1953). The concept of pattern in ecology. *Proceedings of the National Academy of Sciences* 105: 1-12.

Jensen A., B. Rystad, S. Melsom (1974). Heavy metal tolerance of marine phytoplankton. I. The tolerance of three algal species to zinc in coastal sea water. *Journal of Experimental Marine Biology and Ecology* 15: 145-157.

Leland H.V., J.L. Carter (1984). Effects of copper on species composition of periphyton in Sierra Nevada, California, stream. *Freshwater Biology* 14: 281-296.

Levin S.A. (1992). The problem of pattern and scale in ecology. *Ecology* 73: 1943-1967.

Loftin, I.R., Franke, S., Roberts, S.A., Weichsel, A., Heroux, A., Montfort, W.R., Rensing, C. & McEvoy, M.M. (2005). A novel copper-binding fold for the periplasmic copper resistance protein CusF. *Biochemistry* 44, pp. 10533-10540..

Macomber, L., Rensing, C. & Imlay, J.A. (2007). Intracellular copper does not catalyze the formation of oxidative DNA damage in Escherichia coli. *J. Bacteriol.* 189, pp. 1616-1626.

Macomber, L. & Imlay, J. (2009). The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. *Proc. Natl. Acad. Sci. U. S. A.* 106, pp. 8344-8349.

May R.M. (1977). Thresholds and breakpoints in ecosystems with a multiplicity of stable states. *Nature* 269: 471-477.

Medley C.N., W.H. Clements (1998). Responses of diatom communities to heavy metals in streams: the influence of longitudinal variation. *Applied Ecology* 8: 631-644.

Mooney H.A., Godron M. (1983). Disturbance and ecosystems. Components of response. Springer-Verlag Berlin, Heidelberg, Germany. *Hydrobiologia: Acta Hydrobiology, Limnology et Protistology*, 249 p.

Munees A., A. Malik. (2012). Bioaccumulation of heavy metals by zinc resistant bacteria isolated from agricultural soils irrigated with wastewater. *Bacteriology Journal* 2: 12-21.

Olafsen, J.A. (1993). The microbial ecology of fish aquaculture. In: Heen, K., Monahan, R.L., Utter, F. ŽEds., Salmon Aquaculture: An overview of recent research. Blackwell, Fishing News Books, Oxford, pp. 166–175.

Olafsen J.A. (2001). Interactions between fish larvae and bacteria in marine aquaculture. *Aquaculture* 200: 223–247.

Outten, F.W., Huffman, D.L., Hale, J.A. & O'Halloran, T.V. (2001). The independent cue and cus systems confer copper tolerance during aerobic and anaerobic growth in Escherichia coli. *J. Biol. Chem.* 276, pp. 30670-30677.

Paulsson M., B. Nyström, H. Blanck (2000). Long-term toxicity of zinc to bacteria and algae in periphyton communities from the river Göta Älv, based on a microcoms study. *Aquatic Toxicology* 47: 243-257.

Patrick R. (1961). A study of the numbers and kinds of species found in rivers of the Eastern United States. *Proceedings of the Academy of Natural Sciences of Philadelphia* 113: 215-258.

Petraitis P.S., S.R. Dudgeon (2004). Detection of alternative stable states in marine communities. *Journal of Experimental Marine Biology and Ecology* 300: 343-371.

Rensing, C., Fan, B., Sharma, R., Mitra, B. & Rosen, B.P. (2000). CopA: An Escherichia coli Cu(I)-translocating P-type ATPase. *Proc. Natl. Acad. Sci. U. S. A.* **97**, pp. 652-656.

Rensing, C. & Grass, G. (2003). Escherichia coli mechanisms of copper homeostasis in a changing environment. *FEMS Microbiol. Rev.* 27, pp. 197-213.

Rietkerk M., M.C. Boerlijst, F. van Langevelde, R. HilleRisLambers, J. van de Koppel, L. Kumar, H.H.T. Prins & de Roos, A.M. (2002). Self-organization of vegetation in arid ecosystems. *American Naturalist* 160: 524-530.

Rietkerk M., S.C. Dekker, P.C. de Ruiter, J. van de Koppel. (2004a). Self-organized patchiness and catastrophic shifts in ecosystems. *Science* 305: 1926-1929.

Rietkerk M., S.C. Dekker, M.J. Wassen, A.W.M. Verkroost, M.F.P. Bierkens. (2004b). A putative mechanism for bog patterning. *American Naturalist* 163: 699-708.

Riquelme C.E., R.E. Avendaño-Herrera (2003). Microalgae and bacteria interaction in the aquatic environment and their potential use in aquaculture. *Revista Chilena de Historia Natural* 76: 725-736.

Rott E. (1991). Methodological aspects and perspectives in the use of periphyton for monitoring and protecting rivers. Em: Whitton B.A., E. Rott, G. Friedrich (eds) *Use of Algae for Monitoring Rivers*. Institut für Botanik, Universität Innsbruck, Innsbruck, Austria, pp. 9-16.

Say P.J., B.A. Whitton (1977). Influence of zinc on lotic plants. II. Environmental effects on toxicity of zinc to *Hormidium rivulare*. *Freshwater Biology* 7: 377-384.

Say P.J., B.A. Whitton (1981). Chemistry and plant ecology of zinc-rich streams in Northern Pennines. Em: Say P.J., B.A. Whitton (eds) *Heavy metals in Northern England: Environmental and Biological aspects*. Univ Durham, Durham, United Kingdom, pp. 55-63.

Scheffer M., S.H. Hosper, M.L. Meijer, B. Moss, E. Jeppesen (1993). Alternative equilibria in shallow lakes. *Trends in Ecology and Evolution* 8: 275-279.

Scheffer M., S. Carpenter, J.A. Foley, C. Folke, B. Walker (2001). Catastrophic shifts in ecosystems. *Nature* 413: 591-596.

Scheffer, M.. J. Bascompte, W.A. Brock, V. Brovkin, S.R. Carpenter, V. Dakos, H.

Held, E.H. van Nes, M. Rietkerk and G. Sugihara (2009). Early-warning signals for critical transitions. *Nature* 461: 53-59.

Soldo D., R. Behra (2000). Long-term effects of copper on the structure of freshwater periphyton communities and their tolerance to copper, zinc, nickel and silver. *Aquatic Toxicology* 47: 181-189.

Stevenson R.J., M.L. Bothwell, , R.L. Lowe (eds). (1996). Algal ecology - Freshwater Benthic Ecosystems. - Academic Pres.

Stevenson R.J., L.L. Bahls (1999). Rapid biosassessment protocols for use in streams and rivers: periphyton, benthic macroinvertebrates and fish. 6. Periphyton protocols. Web site USA Environmental Protection Agency (EPA): www.epa.gov/owowwtr1/monitoring/rbp/ch06main.html#Section6.

Stoermer E.F., J.P. Smol (1999). The diatoms: Applications for the environmental and earth sciences. Stoermer E.F., J.P. Smol (eds), Cambridge University Press, Cambridge, United Kingdom.

Sundar K., Vidya R., Amitava Mukherjee and Chandrasekaran N. (2010). High chromium tolerant bacterial strains from Palar river basin: Impact of tannery Pollution. *Journal of Environmental and Earth Sciences* 2: 112-117.

Takamura N., F. Kasai, M.M. Watanabe (1989). Effects of Cu, Cd and Zn on photosynthesis of freshwater benthic algae. *Journal of Applied Phycology* 1: 39-52.

Tilman D., S.S. Kilham, P. Kilham (1982). Phytoplankton community ecology: The role of limiting nutrients. *Annual Review Ecology System*, 13: 349-72.

Tilman D. (1982). Resource competition and community structure. *Monographs in population biology* 17. Princeton University Press, Princeton, New Jersey, 296 pp.

Torres-Urquidy O., Bright K. (2012). Efficacy of multiple metals against copper-resistant bacterial strains. Journal of Applied Microbiology, Vol: 112(4) pp 695-704.

Turner M.G. (2005). Landscape ecology in North America: Past, present, and future. *Ecology* 86:1967-1974.

Underwood G.J.C., J. Kromkamp. (1999). Primary production by phytoplankton and microphytobenthos in estuaries, *Advances in Ecological Research*, 29: 93-153.

Underwood G.J.C., L. Provot (2000). Determining the environmental preferences of four estuarine epipelic diatom taxa: growth across a range of salinity, nitrate and ammonium conditions. *European Journal of Phycology*, 35:173-182.

Van de Koppel J., R.D. Bardgett, J. Bengtsson, C. Rodriguez-Barrueco, M. Rietkerk, M.J. Wassen, V. Wolters. (2005). The effects of spatial scale on trophic interactions. *Ecosystems* 8:801-807.

Wassens, S. Watts, R.J., Howitt, J., Spencer, J., Zander, A. and Hall, A. (2011) Monitoring of ecosystem responses to the delivery of environmental water in the Murrumbidgee system. Institute for Land, Water and Society. Report 1.

Wiens J.A. (1989). Spatial scaling in ecology. *Functional Ecology*, 3: 385-397.

Xavier J.B., E. Martinez-Garcia, K. R. Foster. (2009). Social evolution of spatial patterns in bacterial biofilms: When conflict drives disorder. *American Naturalist* 174:1-12.