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Programa de Pós-Graduação em Ciências da Engenharia Ambiental

PATRÍCIA CARLA GILONI DE LIMA

Estudo dos mecanismos de detoxificação e tolerância aos metais cromo e cobre em *Pseudokirchneriella subcapitata* e *Pistia stratiotes* e o uso das macrófitas *Typha* sp e *Phragmites* sp na remoção de nutrientes em *wetlands* construídos.

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Tese apresentada à Escola de Engenharia de São Carlos (EESC), da Universidade de São Paulo (USP), para obtenção do título de Doutor em Ciências da Engenharia Ambiental.

Orientador: Prof. Assoc. Evaldo Luiz Gaeta Espíndola

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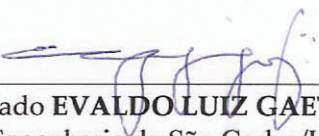
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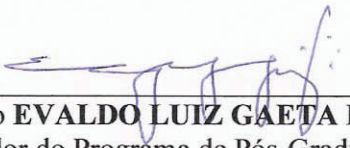
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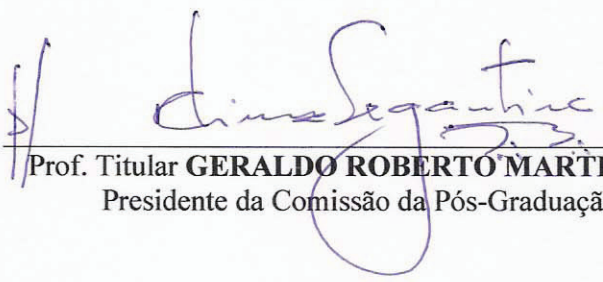
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RESUMO

GILONI-LIMA, P. C. Estudo dos mecanismos de detoxificaçao e tolerância aos metais cromo e cobre em *Pseudokirchneriella subcapitata* e *Pistia stratiotes* e o uso das macrófitas *Typha* sp. e *Phragmites* sp. na remoção de nutrientes em wetlands construídos. 2010. Tese (Doutorado). Programa de Pós-Graduação em Ciências da Engenharia Ambiental. Escola de Engenharia de São Carlos (EESC), Universidade de São Paulo (USP), São Carlos-SP, 2010.

A presente pesquisa teve por objetivos principais: (1) estudar a bioacumulação do metal cromo (40-50 μgL^{-1}) na Clorophyceae *Pseudokirchneriella subcapitata* (Korshikov) Hindak 1990 e dos metais cobre (2-10 μgL^{-1}) e cromo (1-6 mgL^{-1}) na macrófita *Pistia stratiotes* L.; (2) avaliar os mecanismos de detoxificação, as estratégias de defesa e tolerância de *Pistia stratiotes* L., visando recomendar seu uso na fitorremediação; ambos através do uso do Delineamento Composto Central (DCC) e Metodologia de Superfície de Resposta (MSR), e (3) estudar a dinâmica de remoção de nutrientes em *wetlands* construídos, plantados e não plantados com as macrófitas *Typha* sp. e *Phragmites* sp., submetidos a diferentes regimes de fluxo e condições hidráulicas de operação. A bioacumulação de cromo em *P. subcapitata* e sua relação com o biovolume demonstraram uma possível estratégia de detoxificação. *P. stratiotes* desenvolve uma bioacumulação mais intensa nas raízes, resultados que são confirmados pela peroxidação de lipídios e a indução do estresse oxidativo causado pelo cromo. As enzimas catalase e glutationa redutase, induzidas pelo cobre em *P. stratiotes*, também apresentaram atividade mais intensa nas raízes. O teor de clorofila, em geral apresentou aumento nos tempos iniciais e decresceu no decorrer do tempo, em concentrações mais elevadas de cromo e cobre. Na análise da emissão de fluorescência da clorofila, o rendimento fotossintético e o índice de vitalidade foram os parâmetros mais sensíveis ao estresse causado por cromo em *P. stratiotes*. Os resultados obtidos na pesquisa com o DCC e a MSR permitem recomendar seu uso na ecotoxicologia aquática, pois podem gerar modelos preditivos de toxicidade; ampliar a compreensão dos mecanismos de detoxicaçao; reduzir o número de experimentos sem perder a confiabilidade dos dados e reduzir a geração de resíduos. Nos estudos realizados com os *wetlands* construídos, os parâmetros físico-químicos avaliados revelaram variação sazonal durante o período experimental (verão/2007, inverno/2008 e verão/2008). *Typha* sp. e *Phragmites* sp. estão entre as plantas mais comumente utilizadas nos *wetlands* construídos e sua presença amplia as condições de filtração do sistema, mas a eficiência da espécie na remoção dos nutrientes (amônia e fosfato) depende do regime de fluxo e das condições hidráulicas aplicadas. Os sistemas com fluxo subsuperficial com a superfície livre de água foram os *wetlands* que desempenharam melhor *capacidade* na remoção de nutrientes. Uma vez que a poluição dos corpos d´água tem sido um problema constante na atualidade, estudos como estes oferecem subsídios para propostas futuras de preservação e recuperação ambiental, além de ampliar os conhecimentos sobre as macrófitas e sua aplicação na descontaminação ambiental em corpos d´água e em sistemas de depuração de águas residuárias.

Palavras-chave: bioacumulação; planejamento experimental; estresse oxidativo; fluorescência; clorofila; superóxido dismutase; águas residuárias; alagados construídos.

ABSTRACT

GILONI-LIMA, P. C. Study of tolerance and detoxification mechanisms to metals chromium and copper in *Pseudokirchneriella subcapitata* and *Pistia stratiotes,* and the use of macrophytes *Typha* sp. and *Phragmites* sp. in the nutrients removal in constructed wetlands. 2010. Dissertation (Doctorade). Graduate Program in Environmental Engineering Sciences. Engineering Scholl in São Carlos (EESC), University os São Paulo (USP), São Carlos-SP, 2010.

This research had as main objectives: (1) study the bioaccumulation of chromium metal (40-50 µgL-1) in Clorophyceae *Pseudokirchneriella subcapitata* (Korshikov) Hindak 1990 and of copper (2-10 µgL-1) and chromium (1 -6 mgL-1) in the macrophyte *Pistia stratiotes* L. (2) study the mechanisms of detoxification, defense strategies and tolerance of *Pistia stratiotes* L. in order to recommend their use in phytoremediation, both through the use of Central Composite Design (DCC) and Response Surface Methodology (RSM), and (3) study the dynamics of nutrient removal in constructed wetlands, planted and unplanted with macrophytes: *Typha* sp. and *Phragmites* sp. subjected to different flow regimes and hydraulic conditions of operation. The bioaccumulation of chromium in *P. subcapitata* and its relation to biovolume shows a possible strategy for detoxification. *P. stratiotes* develops a more intense bioaccumulation in roots and these results are confirmed by lipid peroxidation and induction of oxidative stress caused by chromium. The enzymes catalase and glutathione reductase induced by copper in *P. stratiotes*, also showed the strongest activity in the roots. The chlorophyll content in general showed an increase in early and decreased over time, in higher concentrations of chromium and copper. In analyzing the fluorescence emission of chlorophyll, the photosynthetic yield and the index of vitality were the parameters most sensitive to stress caused by chromium in *P. stratiotes*. The results obtained in research with the DCC and MSR allowed to recommend their use in aquatic ecotoxicology, because they allow: to generate predictive models of toxicity, the simulation of such models expanding the understanding of the mechanisms of detoxification; reduce the number of experiments without losing the reliability of data and reducing waste generation. In studies with constructed wetlands, the physicochemical parameters evaluated showed seasonal variation observed during the experimental period (summer/2007, winter/2008, summer/2008). *Typha* sp. and *Phragmites* sp. are among the most commonly used plants in constructed wetlands, and its presence extends the conditions of filtration system, but the efficiency of the species in the removal of nutrients (ammonia and phosphate) depends on the flow regime and hydraulic conditions applied in the system. The systems with subsurface flow with free surface water wetlands that have been played better capacidade in removing nutrients. Pollution of water bodies has been a constant problem at the moment, and studies like these provide input for future proposals for the preservation and environmental restoration, in addition to expanding our knowledge on the macrophytes, and its application in environmental remediation in water bodies and systems purification of wastewater**.**

Key-words: bioaccumulation, experimental design; oxidative stress; fluorescence; chlorophyll; superoxide dismutase; wastewater.

SUMÁRIO

CAPÍTULO 1. INTRODUÇÃO E OBJETIVOS

1. INTRODUÇÃO

As aglomerações populacionais e industriais sempre ocorrem nas proximidades ou às margens de rios; desta forma, os processos de urbanização e industrialização têm tornado mais evidentes os problemas de contaminação do ambiente. Atualmente, a poluição aquática causada por efluentes industriais, efluentes de esgotos domésticos, atividades agriculturais, dentre outros, têm elevado consideravelmente a concentração de poluentes no ambiente, afetando de forma diferenciada os diversos compartimentos dos sistemas bióticos. Prasad e Freitas (2003) afirmam que, em geral, todos os compartimentos terrestres (litosfera, hidrosfera e atmosfera) estão poluídos em maior ou menor grau por uma variedade de poluentes inorgânicos e orgânicos provenientes das atividades antropogênicas, alterando a ciclagem biogeoquímica natural.

Os metais pesados estão entre os toxicantes presentes nos efluentes de origem agricultural e industrial e têm representado uma fonte de poluição ambiental que preocupa pesquisadores, órgãos ambientais e órgãos relacionados à saúde pública. Isto ocorre por que os metais não são biodegradáveis e, portanto de natureza cumulativa nos diferentes compartimentos e nos diferentes níveis das cadeias tróficas. Esta cascata de eventos acaba por afetar a vida dos organismos aquáticos e das pessoas que deles se alimentam ou a qualidade da água para aqueles que as utilizam como fonte de abastecimento.

Bioacumulação é o processo que promove aumento gradativo das concentrações de substâncias químicas nos organismos aquáticos em comparação com a água, absorvidas por

diferentes vias e de várias formas (como por exemplo: por ingestão de alimentos, via dérmica, via respiratória). Essas substâncias químicas se concentram ao longo da cadeia alimentar em concentrações cada vez maiores, promovendo a Biomagnificação (MACKAY; FRASER, 2000).

Sendo assim, a avaliação da qualidade ecológica e sanitária da água é de fundamental importância no gerenciamento das águas superficiais, no controle da poluição ambiental e na proteção de ecossistemas aquáticos. Os critérios utilizados para tal avaliação envolvem um grande número de variáveis físicas, químicas e biológicas, necessárias para compreender a complexidade de interações entre os toxicantes químicos e os diversos níveis de organização dos ecossistemas. Entre as diferentes abordagens de estudo, a ecotoxicologia é a ciência que avalia os efeitos adversos destas substâncias químicas liberadas nos ecossistemas, envolvendo testes ou bioensaios de toxicidade.

Os testes de toxicidade ou bioensaios ecotoxicológicos representam importantes ferramentas na compreensão dos efeitos de impactos sobre os compartimentos bióticos, utilizando-se dos organismos vivos como biosensores (CAIRNS et al., 1998). Estes testes podem avaliar a toxicidade de diversas substâncias químicas para uma ou diversas espécies, e a sensibilidade dessas espécies ao agente tóxico, sendo importantes na determinação de concentrações seguras de agentes químicos para a preservação da vida aquática e para a qualidade das águas e sedimentos (ZAGATTO; BERTOLETTI, 2008). Os testes de toxicidade com algas têm sido utilizados na avaliação do potencial de impacto da poluição em ecossistemas aquáticos, permitindo prever possíveis injúrias aos organismos vivos e estabelecer os níveis de tolerância máximos aos toxicantes. O uso de plantas aquáticas nos testes de toxicidade é ainda pouco utilizado, em virtude de representarem testes laboriosos e porque muitas espécies exibem distintos requerimentos ambientais e seu crescimento é lento.

Pode-se dizer que, independentemente do organismo a ser utilizado no bioensaio, sua utilização provê uma estimativa direta e integrada da toxicidade do metal ou do toxicante sob análise (LABRA et al., 2007). Em algas, as respostas mais utilizadas nos bioensaios são avaliações da biomassa, da taxa de crescimento e da densidade celular, ou ainda da atividade metabólica celular.

Um importante fator associado à toxicidade de metais, como o cobre e o cromo, é a indução ou geração de estresse oxidativo em plantas (SINHA et al., 2005; ODJEGBA; FASIDI, 2007; TEWARI et al., 2008; MONFERRÁN, et al., 2009; UPADHYAY; PANDA, 2009). Porém, as plantas geralmente têm sistemas de defesa antioxidante, enzimático e não enzimático que permitem neutralizar as ERO e proteger as células vegetais dos danos oxidativos (GRATÃO et al., 2005). Os níveis de indução e proteção contra o estresse oxidativo parecem estar relacionados com mecanismos de tolerância aos metais, os quais dependem da espécie e do balanço entre os fatores que favorecem e os que reduzem o estresse oxidativo (SINHA et al., 2005; ODJEGBA; FASIDI, 2007).

Em organismos fotossintetizantes, os metais também são capazes de afetar o seu estado fisiológico: alterações na ultrestrutura da membrana do cloroplasto, degradação de pigmentos (clorofila e carotenóides), decréscimo na assimilação de CO_2 e modificação da fluorescência da clorofila a (VAJPAYEE et al., 2000; PANDA; CHOUDHURY, 2005; VERNAY et al., 2007). Desta forma, a fluorescência da clorofila também tem sido um dos métodos utilizados na avaliação e na compreensão dos possíveis mecanismos de toxicidade dos metais pesados em algas e plantas (JUNEAU et al., 2003; PAIVA et al., 2009).

Em plantas terrestres, a ação negativa dos metais em diferentes aspectos fisiológicos do seu desenvolvimento tem sido bem documentada (VERNAY et al., 2007; GANESH et al., 2008; SOBRINO-PLATA et al., 2009), enquanto que para as plantas aquáticas seu potencial

na remoção de metais (fitorremediação) tem recebido mais atenção (KLUMP et al., 2002; MAINE et al., 2004). A fitorremediação é a tecnologia baseada no uso de plantas e microrganismos associados para remover, sequestrar e/ou detoxificar vários tipos de poluentes ambientais da água, do solo, sedimentos e do ar (MEMON; SCHRÖDER, 2009).

As macrófitas são plantas aquáticas que crescem na água ou próximo aos corpos d'água, as quais podem estar imersas, submersas ou flutuantes. São importantes constituintes dos sistemas aquáticos e apresentam um importante papel na biogeoquímica destes ambientes, através da circulação ativa e passiva de diversos elementos (WEIS; WEIS, 2004). Estes organismos representam verdadeiros filtros biológicos, contribuindo para manutenção dos ecossistemas aquáticos, razão que conduz os pesquisadores à compreensão de suas funções no ambiente aquático e seus mecanismos de tolerância em presença dos metais (NIGAM et al., 1998; SANITÀ DI TOPPI et al., 2007).

A despeito do uso potencial das macrófitas na fitorremediação, importantes avanços vêm ocorrendo não só na compreensão do seu comportamento, mas também na ampliação da remoção de nutrientes e sólidos, processos utilizados na otimização dos sistemas de depuração em *weltands* construídos ou artificiais (SUÑE et al., 2007; ZIMMELS et al., 2005). O uso de *wetlands* construídos artificialmente no tratamento de águas residuárias tais como efluentes domésticos, águas de escoamento urbano e das chuvas, efluentes agriculturais e industriais, tem tido bons resultados e sua tecnologia tem sido aplicada e expandida. A motivação que tem recebido esta tecnologia ocorre em função:

(1)   dos fenômenos negativos que representam o acúmulo de N e P nos corpos d´água, provenientes das águas residuárias;

(2)   da crescente necessidade de redução de custos provenientes de combustíveis fósseis; e

(3)   do aumento dos problemas referentes às mudanças climáticas.

Aspectos relevantes como esses, provêm incentivos financeiros e apoio das políticas públicas, por se tratar de implementação de tecnologia "verde" de baixo custo e baixo consumo de energia (LEE et al., 2009).

2. OBJETIVOS

2.1. Objetivo Geral

A presente pesquisa tem por objetivos principais: (1) estudar a bioacumulação do metal cromo na Chlorophyceae *Pseudokirchneriella subcapitata* (Korshikov) Hindak 1990 e dos metais cobre e cromo na macrófita *Pistia stratiotes* L.; (2) avaliar os mecanismos de detoxificação, as estratégias de defesa e tolerância de *Pistia stratiotes* L., ampliando seu uso na fitorremediaçao; ambos através do uso do Delineamento Composto Central (DCC) e Metodologia de Superfície de Resposta (MSR), e (3) estudar a dinâmica de remoção de nutrientes em *wetlands* construídos, plantados e não plantados com as macrófitas *Typha* sp. e *Phragmites* sp., submetidos a diferentes regimes de fluxo e condições hidráulicas de operação.

2.2. Objetivos Específicos

A pesquisa tem como objetivos específicos:

- Avaliar a toxicidade do cromo no crescimento, na biomassa e no biovolume de *Pseudokirchneriella subcapitata* através do DCC e MSR;

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- Avaliar a bioacumulação do cromo em *Pseudokirchneriella subcapitata* e predizer sua toxicidade crônica através da simulação de dados gerados por modelos matemáticos no DCC;

- Avaliar a bioacumulação do cromo em *Pistia stratiotes* e predizer sua toxicidade crônica através da análise da fluorescência da clorofila *a* e do teor de pigmentos fotossintéticos pelo DCC e MSR;

- Avaliar a toxicidade dos metais cromo e cobre em *Pistia stratiotes* através da indução do estresse oxidativo e da peroxidaçao de lipídios, e da bioacumulaçao de metais por meio do CCD e MSR;

- Avaliar a capacidade de remoção de amônia e fosfato nos *wetlands* construídos plantados e não plantados com as macrófitas *Typha* sp. e *Phragmites* sp;

- Avaliar a influência de diferentes regimes de fluxo e condições hidráulicas de operação sobre a capacidade de remoção de amônia e fosfato nos *wetlands* construídos.

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CAPÍTULO 2. ESTRUTURA DA TESE, CONTEXTUALIZAÇÃO E JUSTIFICATIVA DA PESQUISA

2.1. ESTRUTURA DA TESE

A tese foi estruturada na forma de artigos, os quais são apresentados em capítulos, visando facilitar a publicação dos resultados obtidos durante a pesquisa em revistas científicas. Como os artigos serão enviados a diferentes revistas, a forma de apresentação, formatação e citação das referências serão diferenciadas entre os capítulos, incluindo-se ainda a repetição de alguns conteúdos e, principalmente, dos procedimentos metodológicos que são comuns em alguns dos artigos. Desta forma, a partir do capítulo 4 são apresentados, em cada capítulo, os seguintes itens: introdução, material e métodos, resultados, discussões, conclusões e referências bibliográficas. Alguns dos artigos estão apresentados em inglês, uma vez que parte do doutorado foi realizada na Universidade de Leon, na Espanha, sob a supervisão do Prof. Dr. Eloy Mantecón Bécares, o que é permitido pelas normas da pós-graduação da Universidade de São Paulo.

A seguir apresenta-se uma síntese das informações contidas nos diferentes capítulos da tese de doutorado:

1. No capítulo 1 é feita uma introdução ao tema da pesquisa, descrevendo os objetivos gerais e específicos. No capítulo 2 apresenta-se a estrutura da tese, a contextualização da pesquisa e os referenciais teóricos que justificam a realização da pesquisa. As etapas da pesquisa e os procedimentos metodológicos adotados são descritos no capitulo 3 e a partir deste seguem-se os artigos já submetidos e a serem publicados.

2. No capítulo 4 apresenta-se um artigo que já foi aceito para publicação (*in press*) na revista científica *Ecotoxicology*, da Springer-Verlag, de Londres, e nele são descritos os resultados dos ensaios ecotoxicológicos realizados com a alga

Chlorophyceae *Pseudokirchneriella subcapitata* submetida ao metal cromo, através do uso do planejamento experimental.

3. Os resultados obtidos sobre a bioacumulação de cromo em *P. subcapitata* e as relações da bioacumulação com o biovolume algal são apresentados no Capítulo 5, o qual já foi aceito para publicação na revista Journal of the Brazilian Society of Ecotoxicology, da Sociedade Brasileira de Ecotoxicologia (SETAC, Brasil). Apresenta-se, ainda, o uso da simulação da bioacumulaçao e do biovolume algal a partir dos modelos de regressão matemática gerados por essa metodologia estatística, dentro da faixa de concentração de cromo estudada e dos tempos de exposição aplicados.

4. No capítulo 6 a proposta foi utilizar a variação da fluorescência da clorofila *a* através do planejamento experimental e da metodologia de superfície de resposta na avaliação da toxicidade crônica de cromo. O artigo propõe o uso destas ferramentas, mais rápidas e sensíveis na avaliação do estresse em *Pistia stratiotes*, como indicadoras de estresse ambiental causado por cromo.

5. Nos capítulo 7 e 8 buscou-se analisar algumas respostas fisiológicas em *P. stratiotes*, causadas pela exposição aos metais cromo e cobre através do planejamento experimental. Os referidos capítulos discorrem sobre a indução de estresse oxidativo em *P. stratiotes*, causado pelos referidos metais, bem como a peroxidação de lipídios e variações no conteúdo das clorofilas.

6. Os resultados do trabalho desenvolvido no estágio de doutorado sanduíche, na Universidade de León, na Espanha são apresentados no capítulo 9, no qual se discute a remoção de nutrientes em sistemas de depuração de efluentes domésticos ("*wetlands*" construídos). Nos "*wetlands*" construídos objetivou-se a comparação da

remoção de nutrientes em sistemas plantados com as macrófitas *Typha* sp. e *Phragmites* sp. e sem macrófitas plantadas sob diferentes regimes de fluxo e carga.

7. No capítulo 10 apresentam-se as considerações finais da pesquisa, destacando as relações observadas entre o uso do Delineamento Central Composto (DCC) e da Metodologia de Superfície de Resposta (MSR) e os ensaios ecotoxicológicos realizados com *P. subcapitata* e *P. stratiotes*. Discute-se também a representatividade do uso desta metodologia na Ecotoxicologia Aquática.

2.2. CONTEXTUALIZAÇAO DA PESQUISA E JUSTIFICATIVA

Poluentes Ambientais

A contaminação dos corpos d'água por metais é um dos principais problemas ambientais do mundo moderno (MAINE et al., 2004; MIRETZKY et al., 2004; VENAY et al., 2007). Os metais estão incluídos na categoria de poluentes ambientais que permanecem no ambiente por longos períodos (GRATÃO, et al., 2005), pois diferentemente da matéria orgânica, não podem ser degradados e, portanto, se acumulam na água, no solo, nos sedimentos mais profundos e nos organismos vivos (MIRETZKY et al., 2004; NIGAM et al., 1998), sendo potencialmente perigosos aos humanos, animais e plantas (GRATÃO et al., 2005).

Os metais são elementos naturais e muitos deles são componentes essenciais dos ecossistemas (DE VOS et al., 1991; PINTO et al., 2003; RAVEN et al., 1999). O cobre e o zinco, por exemplo, embora sejam tóxicos a níveis pouco acima das concentrações consideradas como essencial, são micronutrientes essenciais à atividade de muitas enzimas e

de parte de moléculas que apresentam papéis chave no transporte fotossintético de elétrons (HE et al., 2005; RAVEN et al., 1999).

As fontes de contaminação por metais envolvem os efluentes industriais, pesticidas, resíduos de mineração e processamento de produtos minerais e resíduos de curtume, além dos esgotos domésticos e dos resíduos sólidos. Os metais nos sistemas de águas superficiais podem ser de fontes naturais ou antropogênicas, sendo que níveis excessivos de metais podem colocar em risco a saúde humana e o meio ambiente (VARDANYAN; INGOLE, 2006).

Os metais estão presentes no ambiente em uma ampla faixa de estados de oxidação e de números de coordenação, aspectos avaliados pela especiação de metais. Os íons metálicos podem ser classificados como cátions, ânions ou neutros, em espécies protonadas e não protonadas, monoméricas e poliméricas, e ainda podem apresentar vários graus de associação com constituintes naturais (KOTÁS; STASICKA, 2000).

No presente estudo procurou-se avaliar os efeitos do cromo em organismos aquáticos. O interesse na especiação do cromo (Cr) é originado no amplo uso deste metal em várias indústrias, tais como a metalúrgica (aço e ligas metálicas ferrosas e não-ferrosas), refratores (cromo e cromo-magnesita) e químicas (pigmentos, eletroporação, curtumes e outros), as quais têm liberado grandes quantidades de resíduos no ambiente contendo cromo, podendo promover efeitos biológicos e ecológicos adversos. O cromo pode ocorrer em vários estados de oxidação variando de Cr^{+2} a Cr^{+6}, sendo os estados trivalente Cr (III) e o hexavalente Cr (VI) os mais comuns e estáveis no ambiente terrestre. As diferenças entre os dois estados de oxidação refletem não só mudanças em suas propriedades físico-químicas, bem como em sua reatividade química e bioquímica (CERVANTES et al., 2001; KOTÁS; STASICKA, 2000).

Diferentemente do cromo, o cobre (Cu) é um metal de transição, essencial e com atividade redox que está envolvido em muitos processos fisiológicos em plantas, pois também

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existe em múltiplos estados de oxidação *in vivo*. Sob condições fisiológicas o Cu existe como Cu^{+2} e Cu^+ (YRUELA, 2005). O Cu age como um elemento estrutural em proteínas regulatórias e participa no transporte de elétrons fotossintético, respiração mitocondrial, respostas de estresse oxidativo, metabolismo da parede celular e sinalização hormonal (RAVEN et al., 1999), os quais são processos fisiológicos característicos dos organismos vivos e/ou de organismos fotossintetizantes.

As plantas requerem Cu como um micronutriente essencial para seu crescimento e desenvolvimento normal, sendo assim, deficiente ou em excesso o Cu pode causar desordens no crescimento e desenvolvimento das plantas por afetar importantes processos fisiológicos, em particular no transporte fotossintético de elétrons (YRUELA, 2005).

Os metais traço, dentre eles, o cromo e o cobre, têm sido encontrados em ecossistemas brasileiros de diversas regiões, tais como os estados de São Paulo (AVELAR et al., 1997; BEVILÁQUA, 1996), Rio de Janeiro (CARVALHO et al., 1999) e Minas Gerais (JORDÃO et al., 1999). No rio Paraíba do Sul, por exemplo, que possui 1.145 km de extensão e drena os três estados mais importantes e mais desenvolvidos do Brasil (Minas Gerais, São Paulo e Rio de Janeiro), a contaminação por metais na região mais baixa do rio parece ser regulada por variação sazonal, nos efeitos de diluição causados por mudanças na fonte de materiais particulados suspensos, conforme mencionado por Carvalho et al. (1999) e Molisani et al. (1999). O comportamento do Fe e do Cu está associado com o escoamento superficial, sendo que o Cu está possivelmente associado com o uso de fungicidas cúpricos em grande escala nas plantações de cana-de-açúcar. O oposto tende a ser observado para Zn, Cr e Mn, os quais refletem sua fonte principal secundária (efluentes urbanos e industriais), porém seu comportamento parece também ser controlado pelo efeito de diluição.

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No estado de Minas Gerais, as intensas atividades de mineração lançam toneladas de resíduos a céu aberto, na água, no solo e sedimento, causando o acúmulo de concentrações consideráveis de metais pesados e elementos tóxicos que penetram no solo, águas subterrâneas e rios, colocando em perigo a qualidade do ambiente (VEADO et al., 2002). Os resultados das análises realizadas por Veado et al (2006) revelaram a contaminação de metais, tais como Cu, Cr, Fe, Hg, Mn, Zn, dentre outros, nos vários segmentos do rio Das Velhas, nas áreas de mineração, nas regiões das fazendas e na ictiofauna, abrangendo uma faixa de aproximadamente 400 km.

Toxicidade por Metais em Algas e Macrófitas

Alguns metais traço são elementos essenciais para as plantas e animais. Entretanto, sob certas condições ambientais, esses elementos podem se bioacumular em concentrações tóxicas, causando danos fisiológicos. Muitos organismos podem bioconcentrar metais traço proporcionalmente às concentrações encontradas no ambiente em que estão inseridos, não possuindo regulação da concentração de cátions em seus tecidos (RAINBOW; PHILIPS, 1993).

Os organismos mais utilizados na avaliação da toxicidade de substâncias são as algas e o zooplâncton. As algas, através da produção primária, representam a base de diversas cadeias alimentares e o zooplâncton constitui o elo entre os produtores primários e consumidores, além de influenciar a ciclagem de nutrientes e outros elementos nos ecossistemas (HOOK; FISHER, 2002; RAINBOW; PHILIPS, 1993). Dentre as espécies mais freqüentemente utilizadas em testes de toxicidade incluem-se invertebrados (*Daphnia*, *Gammarus*, *Brachionus* e *Ceriodaphnia*), peixes (*Poecilia* sp., *Leponis macrochirus*, *Danio rerio*, *Pimephales promelas* e *Oncorhyncus mykiss*) e algas (*Pseudokirchneriella subcapitata*,

Chlorella, *Microcystis* e *Navicula*) (HOFFMAN et al., 1995). O uso de algas como organismo-teste na avaliação da toxicidade aquática de compostos químicos e do aumento na eutrofização tem crescido desde o início da década de 70 (JANSSEN; HEIJERICK, 2003; WARD et al., 2002; KANEKO et al., 2004).

A utilização de algas como bioindicadoras depende da habilidade (resistência ou sensibilidade) destes organismos em responder a tratamentos potencialmente tóxicos (PINTO et al., 2003). Kleine e Lewis (1995) afirmam que grande parte da informação disponível à comunidade científica relativa ao efeito de fitotoxicidade de compostos químicos e outros potenciais toxicantes é baseada em resultados de trabalhos desenvolvidos com um grupo de Chlorophyceae, tais como: *Pseudokirchneriella subcapitata* e várias espécies de *Scenedesmus*. O uso de *Pseudokirchneriella subcapitata* na avaliação da qualidade da água e no biomonitoramento é, portanto, uma prática bastante comum (ABNT, 2005; GUÉGUEN et al., 2003; PARDOS et al., 1998).

Assim como as algas, as macrófitas aquáticas acumulam os metais que elas absorvem do ambiente e estes também têm efeito cumulativo ao longo das cadeias tróficas. Os metais liberados após a decomposição são transmitidos a organismos de níveis tróficos superiores, representando uma via de ciclagem de elementos traços em ecossistemas aquáticos (SINHA et al., 2005; MIRETZKY et al., 2004). As macrófitas são taxonomicamente próximas das plantas terrestres, porém são fanerógamas aquáticas, as quais residem em ambientes completamente diferentes (VARDANYAN; INGOLE, 2006). Na última década, a importância de plantas aquáticas vasculares nos processos de avaliação de risco ambiental tem sido reconhecida (KLEINE; LEWIS, 1995).

Estes organismos não são importantes apenas como indicadores de contaminantes em ecossistemas aquáticos, mas também como rotas de disposição de toxicantes químicos,

movimento e biodisponibilidade (KLEINE; LEWIS, 1995). Na bioacumulação, plantas com alta capacidade de acumulação de metais e uma boa tolerância a altas concentrações de metais por longos períodos de tempo são requisitos necessários à biorremediação (MAINE et al., 2004). *Salvinia herzogii* e *Pistia stratiotes* estão entre as espécies livres flutuantes de grande dispersão e produtividade e ambas têm demonstrado ser eficiente na absorção de cromo (Cr) e cádmio (Cd) (SUÑE et al., 2007).

Sinha et al. (2005) evidenciaram que concentrações tóxicas de Cr em *Pistia stratiotes* causam danos oxidativos observados pelo aumento na peroxidação de lipídios e decréscimo no conteúdo de clorofila e proteínas. O acúmulo de metais nas raízes pôde ser correlacionado positivamente com a maioria dos parâmetros antioxidantes estudados. Os níveis mais elevados de antioxidantes enzimáticos e não-enzimáticos explicam a razão de *P. stratiotes* tolerar níveis mais elevados de Cr.

Satyakala e Jamil (1992) evidenciaram mudanças bioquímicas significativas em tecidos vegetais de *Eichornia crassipes* e *Pistia stratiotes* submetidas à bioacumulação de elevadas concentrações de cromo, tais como a inibição da síntese de clorofila, a qual resultou em perdas na atividade fotossintética e em redução no conteúdo de carboidratos totais em ambas as macrófitas. Mudanças ultra-estruturais no cloroplasto causadas por Cr têm sido observadas em plantas como *Lemna minor*, *Pistia stratiotes* e *Taxithelium nepalense,* conduzindo a inibição da fotossíntese (BASSI et al., 1990; CHOUDHURY; PANDA, 2004).

Respostas Fisiológicas ao Estresse Causado por Metais em Macrófitas

Como conseqüência de uma ampla gama de estresses abióticos, incluindo os metais pesados, a toxicidade causada pelos metais está relacionada à indução de estresse oxidativo

em plantas (OKAMOTO; COLEPICOLO, 1998; PANDA; CHOUDHURY, 2005; WANG; SHI, 2001). Neste caso, os danos causados por uma ampla faixa de diferentes tipos de estresse ambiental, sejam causados por fatores bióticos e ou abióticos, parecem ser direta ou indiretamente causados por Espécies Reativas de Oxigênio (ERO), também denominado estresse oxidativo (ODJEGBA; FASIDI, 2007). Dentre as muitas alterações provocadas nas células vegetais, as membranas celulares perecem ser o primeiro alvo (MONFERRÁN et al., 2009), por serem as ERO altamente destrutivas, atacando diversos componentes das células, tais como: proteínas e lipídios de membrana e ácidos nucléicos (MATSUMURA et al., 2002). Sendo assim, elevações no nível e/ou atividade dos antioxidantes são considerados indiretamente reflexos do aumento nos níveis de formação das ERO (ODJEGBA; FASIDI, 2007). Os metais estão envolvidos em diversos tipos de mecanismos de geração de espécies ativas de oxigênio (EAOs), como o ânion superóxido ($^{\bullet}O^{2-}$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($^{\bullet}OH^-$) (Figura 1). Mas antes que as EROs possam danificar porções significativas da estrutura celular, os organismos desenvolveram uma ampla faixa de mecanismos protetores que servem para removê-las (LEE; SHIN, 2003, PANDA; CHOUDHURY, 2005; SINHA et al., 2009).

Os compostos protetores sintetizados pelos sistemas de defesa antioxidante podem ser divididos em compostos de baixa massa molecular, tais como a glutationa, fenóis, ascorbato, flavonóides, tocoferóis e carotenóides. Dentre os compostos de alta massa molecular estão as enzimas que podem ser ativadas por indução, como superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPX), ascorbato peroxidase (APX), glutationa redutase (GR), tiorredoxina e peroxirredoxina (LEE; SHIN, 2003, PANDA; CHOUDHURY, 2005; SINHA et al., 2009).

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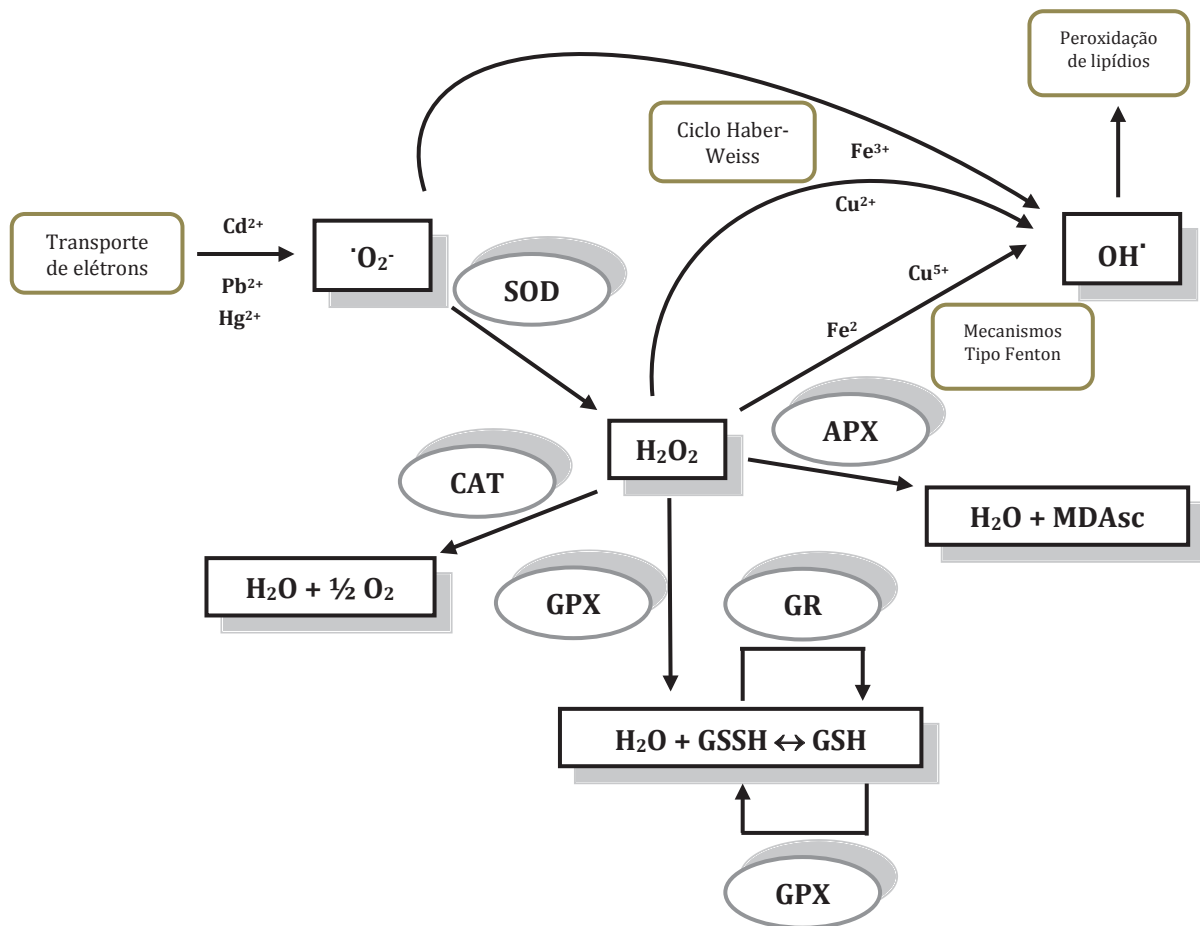
Figura 1. Estresse causado por metais induz a geração de ERO ativando sistema de defesa antioxidante em organismos vivos. SOD (Superóxido dismutase); CAT (catalase); APX (ascorbato peroxidase); GPX (Glutationa peroxidase); GR (Glutationa redutase); GSH e GSSH (forma oxidada e reduzida da glutationa); MDAsc (Monodeidroascorbato). (Modificado de KAPPAS, 1985; PINTO et al., 2003)

A. Sistema Antioxidante

A reação total que envolve a varredura ou "limpeza" do oxigênio ativo - o ânion superóxido ($^{\bullet}O_2^{-}$) - é a foto-redução do dioxigênio à água, via superóxido e peróxido de hidrogênio (H_2O_2) no fotossistema I (PSI) pelos elétrons derivados da água no fotossistema II (PSII). Esse ciclo, definido como ciclo água-água, também promove a dissipação do excesso de energia de excitação sob condições de estresse ambiental. Uma de suas funções

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fisiológicas é proteger as enzimas de varredura, além das enzimas estromais e do complexo PSI, dos danos oxidativos causados pelo $^{\bullet}O_2^{-}$ fotoproduzido no PSI e por outras espécies ativas de oxigênio derivadas deste ânion. Quando o ciclo água-água opera corretamente, os sistemas de varredura são protegidos e enzimas sensíveis ao peróxido de hidrogênio, tais como superóxido dismutase (SOD), ascorbato peroxidase (APX), dentre outras, também são protegidas (ASADA, 1999; SINHA et al., 2005).

Os radicais superóxidos são produzidos pela reação do oxigênio molecular do fotossistema na reação de Meyer. Estes $^{\bullet}O_2^{-}$ são rapidamente convertidos a H_2O_2 pela SOD (Superóxido dismutase) que está associada ao tilacóide com importância relacionada à fotossíntese (ALLEN, 1995). O H_2O_2 pode ser então convertido pela CAT (catalse) ou pela APX (Ascorbato peroxidase) (Figura 1) a H_2O (FADZILLAH et al., 1996).

Segundo Sinha et al (2005) as superóxidos dismutases podem usar vários outros elementos como doadores de elétrons, além do peróxido de hidrogênio, e quanto à localização da enzima, podem estar presentes no vacúolo, na parede celular e nos espaços intercelulares. As peroxidases também podem participar na redução do peróxido de lipídio das membranas do tilacóide a álcool para suprimir a cadeia de oxidação dos fosfolipídios do tilacóide (ASADA, 1999).

O H_2O_2 produzido pela glicolato peroxidase é degradado pela CAT principalmente nos peroxissomos (SCANDALIOS, 1990; AZEVEDO et al., 1998). Segundo Foyer et al. (1994), a CAT está ausente no cloroplasto e a degradação do H_2O_2 nos cloroplastos é feita pela APX ligada à membrana do tilacóide. As moléculas de $^{\bullet}O_2^{-}$ e H_2O_2 que escapam da destruição no tilacóide são destruídas no estroma pela SOD e APX. Radicais monodesidroascorbato produzidos pela APX são convertidos a ascorbato (AA) via ferredoxina (Fd) ou pela enzima Monodesidroascorbato Redutase (MDHAR).

Outra alternativa de reação com a APX é a formação de ácido ascórbico e ácido desidroascorbato, que podem ser convertidos a AA através da enzima Desidroascorbato Redutase (DHAR), que utiliza a GSH como doadora de elétrons. A subseqüente regeneração da glutationa (GSH) requer a participação da GR e nicotinamida adenina dinucleotídeo reduzida (NADPH) (ALLEN, 1995). Estas EAOs são altamente reativas e citotóxicas para todos os organismos, uma vez que elas podem reagir com ácidos graxos insaturados das membranas e promover a peroxidação lipídica.

Desta forma, apesar dos organismos aeróbicos disporem de vantagens energéticas significativas utilizando o oxigênio molecular como um oxidante terminal na respiração, a presença do oxigênio no ambiente celular constitui-se numa ameaça oxidativa constante às suas próprias estruturas e processos devido ao seu potencial de agir como redutor parcial e assim formar as EROs (MALLICK; MOHN, 2000), que podem se tornar altamente destrutivas para células e tecidos se sua produção não for estritamente controlada (RICE-EVANS et al., 1991). Embora, a formação de EROs é uma conseqüência inevitável do metabolismo dos organismos aeróbicos (ANGELOVA et al., 2000) e provocam "estresse oxidativo" devido à sua ação tóxica e mutagênica sobre as células (ANGELOVA et al., 2000; MALLICK; MOHN, 2000).

O estresse oxidativo apresenta um importante papel nos fenômenos de estresse biótico e abiótico, o qual ocorre quando há um sério desbalanço em alguns dos compartimentos celulares entre a produção de ERO e o sistema de defesa antioxidante, conduzindo a sérios problemas fisiológicos celulares (FOYER; NOCTOR, 2000).

Efeitos tóxicos do cromo em algumas plantas aquáticas têm sido relatados por vários autores (KANOUN-BOULÉ et al., 2009; MONFERRÁN et al., 2009; MISHRA et al., 2006). Odjegba e Fasidi (2007) relatam que a exposição de plantas aquáticas aos metais pesados

provoca respostas pronunciadas do sistema antioxidante, os quais protegem as plantas em alguma extensão contra o dano oxidativo, mas a direção da resposta é dependente da espécie de macrófita, do metal utilizado no teste ecotoxicológico e na intensidade do estresse.

Estudos realizados por Sinha et al. (2005) ao observar a relação entre o estresse induzido por cromo e a capacidade antioxidante em *Pistia stratiotes*, sugerem que a capacidade de tolerância destas macrófitas ao cromo depende do balanço que favorece o estresse oxidativo e dos fatores que reduzem o estresse oxidativo.

Upadhyay e Panda (2009) relatam ainda que, exposições de Cu em curto prazo em *Pistia stratiotes*, produzem um efeito notável na fisiologia e bioquímica desta macrófita, induzindo alterações bioquímicas, mudanças na eficiência antioxidante e alterações ultra-estruturais, sugerindo mecanismos de fitotoxicidade ao cobre.

B. Fluorescência da Clorofila

Outro mecanismo comum da ação tóxica de contaminantes é a inibição de processos biológicos tais como a fotossíntese e o transporte de elétrons mitocondrial (BABU et al., 2005). A cinética da fluorescência emitida por algas e plantas superiores é dependente de processos fotossintéticos, bioquímicos e fisiológicos relacionados à fotossíntese (KRAUSE; WEIS, 1984). Por esta razão a fluorescência da clorofila tem sido usada para estudar mecanismos de toxicidade de diferentes contaminantes em algas e plantas superiores (EL JAY et al., 1997, JUNEAU; POPOVIC, 2002;). Além de se tratar de um método simples, rápido, sensível e não destrutivo, é possível estimar vários parâmetros da atividade fotossintética, tais como: fluorescência da clorofila *a*, rendimento fotossintético, *quenching* fotoquímico e não

fotoquímico, são capazes de refletir o estado fisiológico das plantas quando expostas ao efeito tóxico de poluentes ou de estresses ambientais (JUNEAU; POPOVIC, 1999, 2002).

A fluorescência da clorofila fornece informações sobre o estado do fotossistema II (PSII) propiciando uma extensão de uso da energia absorvida pela clorofila e dos danos causados pelo excesso de luminosidade. Danos ao PSII são freqüentemente as primeiras manifestações de estresse nas folhas. A fluorescência pode ser usada para medir a eficiência fotoquímica do PSII (MAXWELL; JOHNSON, 2000; ASADA, 1999).

A fluorescência tem sido proposta como um valioso indicador na investigação dos mecanismos de toxicidade de diferentes poluentes em macro ou microalgas e em plantas superiores em condições de campo ou de laboratório (JUNEAU; POPOVIC, 2002; JUNEAU; POPOVIC, 1999; BOLHÀR-NORDENKAMPF et al., 1989). Contudo, esses parâmetros ainda não foram validados como *end point,* dentre as ferramentas úteis no monitoramento toxicológico de ecossistemas.

Küster e Altenburger (2007) propuseram a validação de um bioensaio baseado na análise de fluorescência pelo método fluorométrico do Pulso de Amplitude Modulada (Imaging-PAM) para espécies de macrófitas aquáticas. O trabalho foi desenvolvido com *Chara canescens* e *Lemna minor* submetidas ao tratamento com os herbicidas atrazina, prometrin e isoproturon e os resultados de CE50 obtidos apresentaram boa correlação com as medições de fluorescência. Desta forma, o Imaging-PAM se revela como uma ferramenta promissora, permitindo uma rápida na varredura dos efeitos químicos em grandes quantidades de amostras com pouco tempo e material (aspectos geralmente negativos nos ensaios que envolvem macrófitas). Porém os autores afirmam que para validar o uso desta metodologia são necessários experimentos com uma ampla gama de toxicantes e seu modo de ação em relação à inibição do PSII.

O efeito fitotóxico dos metais no crescimento vegetal tem sido amplamente estudado em muitas espécies vegetais de interesse agronômico, plantas aquáticas, briófitas e algas (PANDA; CHOUDHURY, 2005). A degradação dos pigmentos clorofilianos pode eventualmente diminuir a eficiência fotossintética nas plantas, os quais pode refletir numa redução do seu crescimento (UPADHYAY; PANDA, 2005).

O acúmulo de metais em macrófitas aquáticas frequentemente induz a importantes distúrbios metabólicos e particularmente à degradação da clorofila (PRASAD et al., 2001; PERALES-VELA et al., 2007). A degradação da clorofila e a inibição da fotossíntese podem ser consideradas como uma resposta específica aos metais, que devem causar uma inibição da α-desidrogenase ácida aminolevulínicaα (-ALAD), uma importante enzima da via biossintética da clorofila (OUZOUNIDOU, 1993). A redução do conteúdo de pigmentos pode ocorrer também devido a peroxidação lipídica da membrana do cloroplasto por ação das espécies reativas de oxigênio induzida no estresse causado por metais (SANDAMANN; BÖGER, 1980).

Reduções no conteúdo de pigmentos fotossintéticos têm sido relatadas em *Eichornia crassipes* e *Hydrilla verticillata* submetidas a tratamento com cobre (LEWIS, 1993); em *Nymphaea alba* submetida a estresse causado por cromo (VAJPAYEE et al., 2000) e em diferentes populações de *Lemna minor* submetida a estresse por cobre (KANOUN-BOULÉ et al., 2009).

Embora, aumento e decréscimo no conteúdo de clorofila tenham sido relatados em diversas espécies vegetais expostas ao Cr VI (SHARMA; SHARMA, 1993; SAMANTARAY

et al., 2001), em geral o Cr VI afeta adversamente o conteúdo de pigmentos fotossintéticos em várias plantas aquáticas (SINGH; SINHA, 2005, CHOO et al., 2007; GANESH et al., 2008).

Considerações sobre Delineamento Composto Central e a Metodologia de Superfície de Resposta

A maioria dos delineamentos experimentais é gerada de maneira que um parâmetro é variado enquanto os outros permanecem constantes. Existem duas desvantagens neste tipo de delineamento: (1) o efeito de um único parâmetro é estudado; (2) a possibilidade de interação entre os diferentes parâmetros não pode ser avaliada (HEIJERICK et al., 2003). O Delineamento Composto Central (DCC) ou planejamento fatorial aplicado neste tipo de delineamento gera um volume de informações no efeito direto dos parâmetros testados e suas interações, enquanto testa um número mínimo de combinações (EDGINTON et al., 2004; HEIJERICK et al., 2003). Mais especificamente, o planejamento experimental auxilia o pesquisador a verificar se mudanças nos valores dos fatores produzem uma variação estatisticamente significativa na resposta observada (FURLANETTO et al., 2003).

Na toxicologia ambiental aquática, onde muitos fatores podem interagir para alterar a toxicidade química, a análise da superfície de resposta também pode ser utilizada para fornecer informações sobre uma combinação de níveis de dois ou mais fatores (EDGINTON et al., 2004). Sun et al. (2009), trabalhando com as interações entre estrogênios químicos em misturas binárias induzidas por vitelogenina, demonstraram a habilidade do planejamento experimental para detectar estes tipos de interações, permitindo avaliações relevantes dos efeitos biológicos no ambiente envolvendo misturas químicas mais complexas.

O uso sistemático da estatística de planejamento experimental no desenvolvimento de uma metodologia pode assegurar delineamentos, suportes para a validação de modelos preditivos e confirmar a validação destes métodos com maior representatividade (FURLANETTO et al., 2003). Modelos preditivos de toxicidade, gerados a partir de relações matemáticas desenvolvidas na aplicação do DCC e MSR (Metodologia de Superfície de Reposta), revelaram sua aplicação nos estudos realizados por Heijerich et al. (2003) e De Schamphelaere et al. (2003). Heijerick et al. (2003) demonstraram que a toxicidade do zinco (Zn) para *D. magna* é dependente do pH, da dureza e da concentração de carbono orgânico dissolvido, sendo que o modelo desenvolvido junto com outras informações já relatadas na literatura conduziriam a uma avaliação de risco para metais mais embasada cientificamente. Resultados semelhantes foram obtidos para os ensaios realizados com *P. subcapitata* e cobre, onde De Schamphelaere et al. (2003) relatam que o modelo desenvolvido apresenta alta capacidade preditiva contribuindo para ampliar a relevância ecológica nos procedimentos de avaliação de risco.

Apesar de encontrarmos resultados promissores na utilização do DCC e MSR na ecotoxicologia aquática, envolvendo modelos preditivos de toxicidade em *D. magna* e *P. subcapitata* submetidas a estresse com metais, nada ainda foi realizado neste sentido com *Pistia stratiotes*.

Um estudo de modelagem multivariada (utilizando análise cluster, análise de discriminante, análise de componentes principais dentre outros) foi desenvolvido com *P. stratiotes* submetida a estresse com cromo. A técnica forneceu informações de um padrão diferencial no início e na magnitude do estresse oxidativo induzido na raiz e parte aérea das plantas expostas em função da concentração de cromo e do tempo de exposição. Exemplos como este tem emergido no sentido de gerar modelos de análise e interpretação de grandes conjuntos de dados com inter-relações complexas entre as variáveis e também tem sido

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aplicado com sucesso em vários estudos de caso: ambientais, químicos, biológicos e ecotoxicológicos (SINHA et al., 2009; SINGH et al., 2004).

Uso de Macrófitas em Sistemas de Depuração de Efluentes Domésticos

A maioria dos estudos de bioacumulação de poluentes em macrófitas é de avaliação na eficiência de remoção de nutrientes ou de efeitos tóxicos observados nestes organismos, uma vez que a eutrofização e o aumento da toxicidade continuam sendo um dos mais importantes problemas relacionados aos recursos hídricos, principalmente em função do elevado aporte de nitrogênio, fósforo, metais e pesticidas, provenientes de fontes diversas como efluentes domésticos, industriais, de agricultura, dentre outros (ZHANG et al., 2008). Esses conhecimentos são importantes não só para a compreensão do comportamento das macrófitas, mas também na otimização dos processos de depuração de efluentes por meio de *wetlands* artificiais (NIGAM et al., 1998; SUÑE et al., 2007).

Os aspectos bioquímicos e de biorremediação também têm sido estudados na macrófita aquática *Pistia stratiotes* L. (TARLYN et al., 1998). Os trabalhos realizados com *P. stratiotes* referem-se à sua distribuição, composição química e sua utilização em reservatórios construídos para tratamento de efluentes. A utilização dessas macrófitas como biorremediadores no tratamento de águas residuárias e como indicadoras de contaminação ambiental são mais comuns que seu uso em testes de toxicidade, onde a bioacumulação é o fator mais rotineiramente investigado (KLUMPP et al., 2002; MAINE et al., 2004).

O sistema de *wetlands* construídos representa uma alternativa ao sistema convencional de tratamento de efluentes domésticos, apresentando baixo custo (LEE et al., 2009) e podendo ser utilizado para tratamento primário, secundário ou terciário (CALHEIROS et al., 2007). Os

wetlands construídos são definidos como sistemas desenhados e produzidos pela engenharia para utilizar os processos que ocorrem em um alagado natural, envolvendo as relações da vegetação, do solo e das associações microbianas para o tratamento de efluentes domésticos (VYMAZAL, 2007).

Considerando que os poluentes são removidos por uma variedade de processos físico-químicos e biológicos nos *wetlands*, numerosos fatores ambientais podem influenciar na remoção de nutrientes. Os principais fatores são temperatura, tempo de retenção hidráulico, tipo e densidade de vegetação, as características das comunidades microbianas, o clima, dentre outras (LEE et al., 2009).

Os *wetlands* construídos possuem uma classificação básica (Figura 2), dependendo do tipo de macrófita em crescimento no sistema, que incluem macrófitas livres flutuantes, macrófitas emergentes enraizadas e macrófitas submersas; e do tipo de regime de fluxo de água, podendo ser de água livre na superfície com fluxo superficial (FWS) e de fluxo subsuperficial (SSF ou HSF) (BRIX; SCHIERUP, 1989).

Nitrogênio e fósforo são nutrientes chaves no ciclo de vida dos *wetlands*, que geralmente provêm amplos benefícios no tratamento de efluentes domésticos, e têm oferecido remoção potencial de nitrogênio (LEE et al., 2009). Tal remoção é obtida através de processos físico-químicos e por técnicas de tratamento biológico. As concentrações de nitrogênio quando elevadas são freqüentemente assunto de interesse, devido a seu potencial de causar efeitos adversos nos sistemas de recepção de água. A remoção biológica de nitrogênio da água e de efluentes domésticos compõe-se primariamente de uma combinação de nitrificação aeróbica e denitrificação anaeróbica (VYMAZAL, 2007).
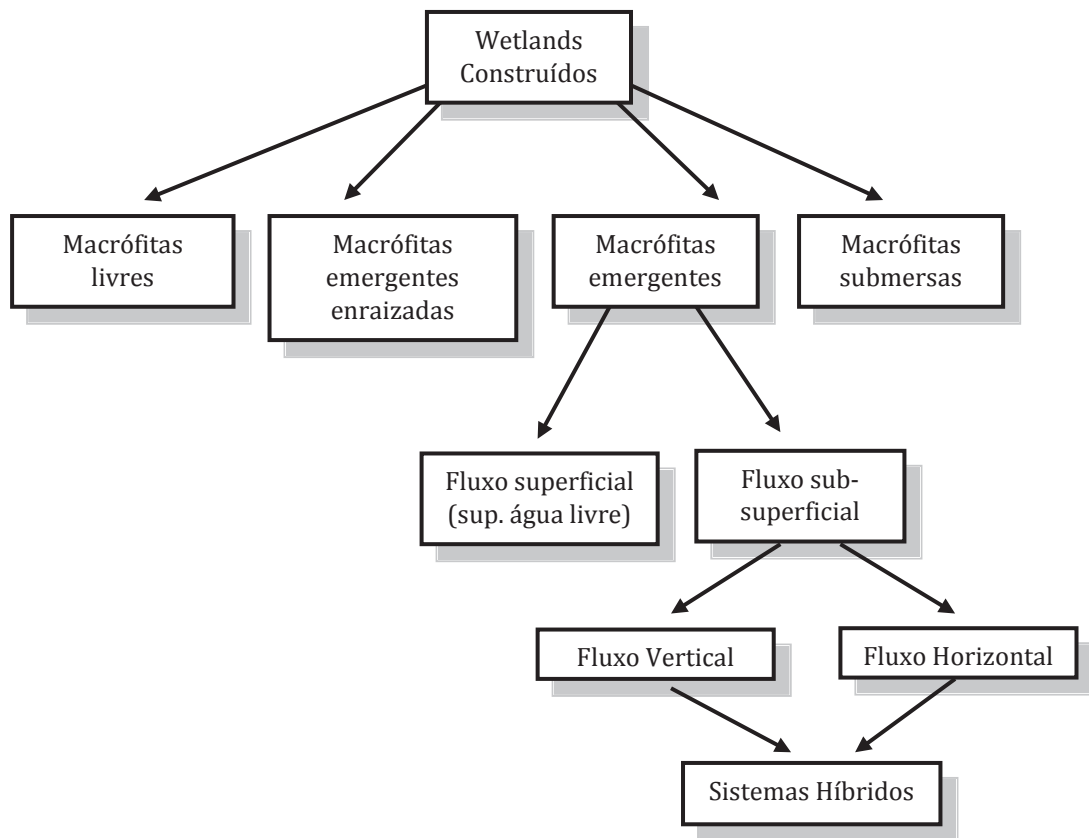
Figura 2. Classificação dos wetlands construídos para tratamento de efluentes domésticos. (Adaptado de VYMAZAL, 2007)

O nitrogênio tem um ciclo biogeoquímico complexo, com múltiplas transformações bióticas e abióticas, envolvendo sete estados de valência (+5 a -3). Os compostos formados incluem uma variedade de formas de nitrogênio orgânico e inorgânico (Tabela 1) que são essenciais a todas as formas de vida biológicas.

O ciclo do fósforo no solo é diferente do ciclo do nitrogênio, não há mudanças de valência durante a assimilação biótica do fósforo inorgânico ou durante a decomposição do fósforo orgânico pelos microrganismos. O fósforo nos *wetlands* ocorre como compostos orgânicos e inorgânicos de fosfato. O ortofosfato livre é a forma do fósforo que se acredita ser utilizada diretamente por algas e macrófitas e representam uma ligação entre a ciclagem das formas orgânica e inorgânica nos *wetlands* (VYMAZAL, 2007).

Sendo assim, as transformações do fósforo durante o tratamento dos efluentes domésticos incluem adsorção, desorção, precipitação, dissolução, absorção microbiana e vegetal, fragmentação, mineralização, sedimentação e decomposição. O fósforo é removido primeiramente por reações de troca com ligantes, onde o fosfato desloca água ou hidroxilas da superfície dos óxidos hidroxilados de Fe e Al. Contudo o meio utilizado para wetlands com fluxo sub-superficial (por exemplo, cascalhos, pedras trituradas) geralmente não contém grandes quantidade de Fe, Al ou Ca e, portanto, a remoção de fósforo é geralmente baixa.

Macrófitas são consideradas os principais componentes biológicos dos *wetlands*. Além de absorver os nutrientes dos efluentes domésticos e diretamente do substrato, elas também agem como catalisadoras nas reações de purificação por elevar a diversidade ambiental da rizosfera e promover uma variedade de reações químicas e biológicas que ampliam a purificação nos *wetlands* construídos (COLEMAN et al., 2001; JENSSEN et al., 1993).

Tabela 1. Transformações do nitrogênio nos *wetlands* construídos.

Processo	Transformação
Volatilização	amônia-N (aq)→ amônia-N (g)
Amonificação	N-orgânico → amônia-N
Nitrificação	amônia-N → nitrito-N → nitrato-N
Nitrato-Amonificação	nitrato-N → amônia-N
Denitrificação	Nitrato-N → nitrito-N → $N_{2(g)}$, N_2O
N_2-fixação	$N_2(g)$ → amônia-N (N-orgânico)
Absorção vegetal/microbiana (Assimilação)	amônia-N, nitrito-, nitrato-N→N-orgânico
Adsorção de amônia	
Nitrogênio orgânico proveniente de processos de decomposição	
Oxidação anaeróbia da amônia	amônia-N→ $N_{2(g)}$

Fonte: Vymazal, 2007.

As plantas apresentam um importante papel na remoção de poluentes nos *wetlands* construídos (BRIX, 1994). Elas não apenas absorvem nutrientes, mas também são capazes de adsorver e acumular metais. As espécies, *Phragmites australis* e algumas espécies de *Cyperus* são comumente utilizadas nos *wetlands* construídos (GREENWAY; WOOLLEY, 1999). Experimentos com outras espécies de macrófitas, tais como: *Phragmites, Iris, Typha* e

Scirpus, demonstraram que em sistemas de tratamento de fluxo superficial para tratamento de efluentes domésticos diluídos, o aspecto que influencia de maneira significativa o rendimento do processo de tratamento é a presença de vegetação nestes sistemas (ANSOLA et al., 1995). Por outro lado, em sistemas de fluxo sub-superficial, os experimentos demonstraram que a vegetação empregada (*Scirpus lacustris*) teria uma função representativa no rendimento do processo, tanto na eliminação da contaminação química, como na contaminação microbiológica (SOTO et al., 2000).

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CAPÍTULO 3. METODOLOGIA DA PESQUISA

A pesquisa foi dividida em três etapas: (1) avaliação da bioacumulação do cromo (Cr) através de ensaios ecotoxicológicos realizados com a Chlorophyceae *Pseudokirchneriella subcapitata*; (2) avaliação da bioacumulação e indução do estresse oxidativo e da fluorescência pelos metais cromo (Cr) e cobre (Cu) através de ensaios ecotoxicológicos realizados com a Araceae *Pistia stratiotes* e (3) avaliação da remoção de nutrientes pelas macrófitas *Typha* sp. e *Phragmites* sp. em "*wetlands*" construídos sob diferentes regimes de fluxo.

Para uma melhor compreensão da estrutura das etapas (1) e (2) da pesquisa, apresenta-se na figura 1 um fluxograma dos parâmetros avaliados.
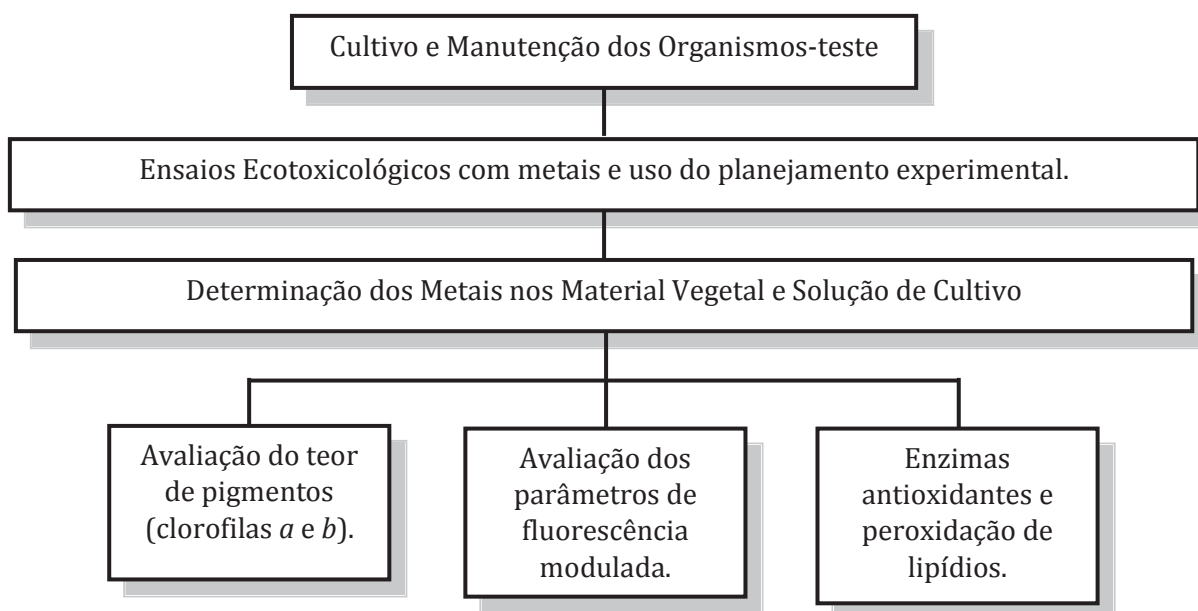
Figura 1. Fluxograma dos parâmetros avaliados e utilizados na execução das etapas (1) e (2) da pesquisa.

O Delineamento Central Composto (DCC), também denominado planejamento experimental, e a Metodologia de Superfície de Resposta (MSR), foram utilizados nos ensaios ecotoxicológicos realizados nas etapas (1) e (2) da pesquisa. Esta metodologia permite considerar simultaneamente vários fatores em diferentes níveis e as interações entre eles, utilizando um pequeno número de experimentos (BARROS NETO et al., 2001). As técnicas de planejamento experimental geralmente são usadas para compreender o efeito de muitas variáveis num sistema por um modelo matemático bem definido (FURLANETTO et al., 2003) podendo ainda ser aplicadas técnicas de inferência estatística para estimar a importância de fatores individuais, a sensibilidade da resposta para cada fator e a magnitude do erro experimental (BARROS NETO et al., 2001).

Diante do exposto, para aplicar a Metodologia de Superfície de Resposta é necessário realizar primeiramente ensaios através de um Delineamento Composto Central. Este método consiste na seleção de um número fixo de níveis para cada um dos fatores ou variáveis de entrada e então executar experimentos com todas as possíveis combinações. A primeira etapa normalmente é utilizada num planejamento fatorial de dois níveis (nível −1 e nível +1) para cada variável. Assim, para k variáveis envolvidas no estudo, o número de experimentos que deve ser realizado para investigar todas as combinações possíveis é igual a 2^k. Geralmente também se faz três ensaios no ponto central (nível 0) para permitir o cálculo do erro experimental (erro puro) (BARROS NETO et al., 2001).

O DCC para as variáveis codificadas x1 e x2 e variáveis selecionadas: X1 = t (tempo em horas) e X2 = C (concentração dos metais cromo em μgL^{-1} ou mgL^{-1}; e cobre em μgL^{-1}), nos níveis fixados para *P. subcapitata* e *P. stratiotes,* são apresentados na tabela 1 e 2, respectivamente. O planejamento fatorial foi 2x2, constituído por 11 experimentos e um controle com três repetições.

Tabela 1. Planejamento fatorial 2^2 para os ensaios ecotoxicológicos de com *P. subcapitata* submetidas ao tratamento com o cromo.

| Experimento | C ($\mu g\ L^{-1}$) | t (h) | C ($\mu g\ L^{-1}$) | t (h) |
	x1	x2	X1	X2
1	-1	-1	41,5	96
2	1	-1	48,5	96
3	-1	1	41,5	168
4	1	1	48,5	168
5	0	0	45,0	132
6	0	0	45,0	132
7	0	0	45,0	132
8	-1,41	0	40,0	132
9	1,41	0	50,0	132
10	0	-1,41	45,0	81
11	0	1,41	45,0	183

x1, x2 = variáveis codificadas (utilizadas nos diferentes níveis do planejamento experimental); X1, X2= variáveis reais (utilizadas na pesquisa, C (concentração) e t (tempo de exposição ao metal).

Tabela 2. Planejamento fatorial 2^2 para os ensaios ecotoxicológicos de com *P. stratiotes* submetidas ao tratamento com os metais: cobre (μg L^{-1}) e cromo (mg L^{-1}).

Experimento	C (μg L^{-1}/ mgL^{-1})	t (h)	C (Cu μgL^{-1})	C (Cr mgL^{-1})	t (h)
	x1	x2	X1	X1	X2
1	-1	-1	3,2	1,7	19
2	1	-1	8,8	5,3	19
3	-1	1	3,2	1,7	53
4	1	1	8,8	2,3	53
5	0	0	6,0	3,5	36
6	0	0	6,0	3,5	36
7	0	0	6,0	3,5	36
8	-1,41	0	2,0	1,0	36
9	1,41	0	10,0	6,0	36
10	0	-1,41	6,0	3,5	12
11	0	1,41	6,0	3,5	60

x1, x2 = variáveis codificadas (utilizadas nos diferentes níveis do planejamento experimental); X1, X2= variáveis reais (utilizadas na pesquisa, C (concentração) e t (tempo de exposição ao metal).

3.1.1. Ensaios Ecotoxicológicos com *Pseudokirchneriella subcapitata*

Cultivo e inoculação de *Pseudokirchneriella subcapitata*

Células de *P. subcapitata* foram obtidas de culturas mantidas no Laboratório de Ecotoxicologia e Ecofisiologia de Organismos Aquáticos do Centro de Recursos Hídricos e Ecologia Aplicada da Universidade de São Paulo. As algas verdes foram cultivadas em meio L. C. Oligo (AFNOR, 1980), as quais foram autoclavadas a 121 ^{o}C por 15 min. em frascos de Erlenmeyer de 2L contendo 1L do meio, cuja composição pode ser observada na Tabela 3.

Após esse procedimento o meio foi inoculado com a concentração aproximada de células algais de 1×10^4 células mL^{-1}. A cultura foi mantida sob aeração constante a 1500 lux, com fotoperíodo de 12:12 h de luz e escuro, à $23^\circ C \pm 2$, até a fase de crescimento exponencial (ABNT, 2005).

Tabela 3. Composição do meio de cultura L. C. Oligo (AFNOR, 1980).

Solução estoque	Composto	Concentração (M)	Volume (mL) requerido da solução estoque
1	$Ca(NO_3)_2 .4H_2O$	0,24	1,00
2	KNO_3	0,99	1,00
3	$MgSO_4 .7H_2O$	0,20	1,00
4	K_2HPO_4	0,23	1,00
5	$CuSO_4 .5H_2O$	0,00016	0,50
	$(NH_4)_6Mo_7O_{24}.4H_2O$	0,00005	
	$ZnSO_4.7H_2O$	0,0002	
	$CoCl_2.6H_2O$	0,0001	
	$Mn(NO_3)_2. 4H_2O$	0,0002	
	$C_6H_8O_2.H_2O$	0,0005	
	H_3BO_3	0,001	
6	$C_6H_5FeO_7.5H_2O$	0,005	0,50
	$FeSO_4.7H_2O$	0,002	
	$FeCl_3.6H_2O$	0,002	
7	$NaHCO_3$	0,2	1,00

Testes de toxicidade

Os testes de toxicidade foram realizados em frascos de Erlenmeyers de 250 mL, previamente autoclavados a 121 $^\circ C$ por 15 min. com 100 mL do meio teste (composição na tabela 3). Antes da inoculação a capela foi esterilizada sob luz ultravioleta por 30 minutos, e posteriormente foram adicionadas as soluções nominais de cromo nas concentrações estabelecidas pelo planejamento experimental (tabela 1). Em seguida, cada uma das soluções

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teste foi inoculada com uma concentração aproximada de 10^4 céls mL^{-1} de células de *P. subcapitata* (ABNT, 2005). Os testes de toxicidade estáticos foram conduzidos nas mesmas condições descritas na manutenção do cultivo no item anterior. As soluções teste foram mantidas em mesa agitadora, sob iluminação contínua (1500 lux) com fotoperíodo de 12:12h a 23±2 °C e sob aeração constante por 81, 96, 132, 168 e 183 horas. Nos tempos de exposição determinados e ao final do teste, de cada erlenmeyer foi retirada uma alíquota para a contagem do número de células, as quais foram preservadas com lugol para posterior contagem.

Densidade, Biomassa e Biovolume algal

O crescimento algáceo foi avaliado através da contagem do número de células (densidade algal) da suspensão em microscópio óptico, utilizando-se câmaras de contagem de Neubauer, após 81, 96, 132, 168 e 183 horas de exposição, e a CE(I)50 calculada através do método Trimmed Spearman – Karber (HAMILTON et al., 1977), a partir do qual foi avaliado a taxa de crescimento populacional e de sua expressão em porcentagem de inibição do crescimento algal em relação ao controle.

A biomassa algal foi estimada a partir das análises de clorofila a. Amostras (5-10 mL) de cada frasco foram filtradas em filtro de membrana (0,45 µm) e a extraçao foi realizada em etanol 80% em ebulição por alguns minutos e posteriormente triturado em almofariz e pistilo. O material triturado foi lavado com etanol (80%), centrifugado e as medidas da absorbância a 665 e 750 nm foram obtidas em espectrofotômetro (F600, FEMTO, USA) contra uma cubeta de referência preenchida com etanol 90% (NUSCH, 1980). Todas as amostras foram analisadas em triplicata

O biovolume algal foi calculado através da medição de pelo menos 30 indivíduos por amostra, das dimensões lineares que incluem as variadas formas algais e equações de regressão matemática para integração de área dos diferentes formatos das algas (HILLEBRAND et al., 1999), em microscópio óptico (Carl Zeiss, modelo 25).

Determinação dos Metais em *P. subcapitata*

Ao final de cada tempo de exposição, amostras provenientes de cada tratamento com cromo de *P. subcapitata* foram analisadas para determinar a concentração de metal acumulado pelas algas. As soluções teste foram filtradas em filtro de membrana Millipore (0.45 μm) e os filtros após secos foram submetidos à digestão ácida com HNO_3 e H_2O_2 (APHA, 1995). Para cada amostra digerida, três filtros limpos foram digeridos e analisados como branco (VAN LOON, 1985). A concentração do metal, expressa em μg Cr mg^{-1} de peso seco das algas foi considerado como a quantidade total de metal acumulado pelas células algais. Todas as amostras foram analisadas em triplicada em espectrometria de absorção atômica (AAS) em chama ou com atomização eletrotérmica em forno de grafite (GFAAS), e espectrometria de massa, no laboratório do Núcleo de Estudos e Ecossistemas Aquáticos (NEEA).

3.1.2. Ensaios ecotoxicológicos com *Pistia stratiotes*

Cultivo e manutenção de *P. stratiotes*

As plantas de *P. stratiotes* provenientes da bacia hidrográfica do Itanhém foram doadas pelo Prof. Dr. Antonio Fernando Monteiro Camargo da Universidade Estadual Paulista

(UNESP) de Rio Claro e foram cultivadas no Centro de Recursos Hídricos e Ecossistemas Aquáticos (CRHEA) da Universidade de São Paulo em São Carlos.

O cultivo foi estabelecido em caixas de água com capacidade de 1.000 L, às quais foram adicionados 45 L de terra vegetal e 400 g de adubo (N:P:K, na proporção de 14:4:8) diluídos em aproximadamente 800 L de água de torneira. O pH foi mantido em torno de 7,0 sendo ajustados com o uso de NaOH 1N.

Testes de Toxicidade com *P. stratiotes*

Nos testes de toxicidade com *P. stratiotes* foram utilizados indivíduos que apresentaram massa entre 8 a 10g de peso fresco. As plantas foram aclimatas em solução nutritiva e posteriormente os testes foram montados em recipientes plásticos com 2 litros de capacidade, e em cada recipiente foram colocados três indivíduos em potes plásticos de 1 litro de solução nutritiva, cuja composição pode ser observada na tabela 4. Solução nutritiva foi completada diariamente para compensar as perdas por transpiração, amostragem e evaporação (ODJEGBA; FASIDI, 2004). Os tempos de exposição e as concentrações de cromo e cobre utilizadas nos testes de toxicidade podem ser observadas na tabela 2.

Tabela 4. Composição da solução nutritiva de utilizada nos testes de toxicidade com *P. stratiotes* (ODJEGBA; FASIDI, 2004).

Micronutrientes	Concentração (mM)	Macronutrientes	Concentração (mM)
H_3BO_3	0,05	KNO_3	1,50
$MnCl_2.4H_2O$	0,01	$Ca(NO_3)_2.4H_2O$	1,25
$ZnSO_4.7H_2O$	0,70	KH_2PO_4	0,50
$CuSO_4.5H_2O$	0,30	$MgSO_4.7H_2O$	0,50
$Na_2MoO_{24}.2H_2O$	0,20		
NaFeEDTA (10%Fe)	0,50		

Determinação dos Metais nos Tecidos Vegetais de *P. stratiotes*

Ao final de cada um dos 11 experimentos, as plantas foram lavadas com água destilada e secas, separadas em raiz e parte aérea e pesadas para obtenção da massa fresca e mantidas a 70 ºC por dois dias ou até peso constante, para determinação da massa seca.

As amostras secas de raiz e folha das macrófitas de cada experimento foram digeridas com HNO_3 8M em béqueres tampados com vidro de relógio. Após a digestão, as amostras foram ressuspendidas e diluídas em HNO_3 diluído. A digestão foi acompanhada dos brancos e a perda de metal foi avaliada através de ensaios de recuperação do metal, nas mesmas condições utilizadas para os experimentos. Foi realizada também a determinação dos metais nas amostras da solução-teste.

As análises dos metais Cr e Cu foram realizadas em espectrometria de absorção atômica (AAS) em chama ou com atomização eletrotérmica em forno de grafite (GFAAS), e espectrometria de massa, no laboratório do Núcleo de Estudos e Ecossistemas Aquáticos (NEEA).

Teor de Clorofilas *a* e *b*

Amostras de folhas da macrófita *P. stratiotes* quando completamente expandidas foram utilizadas para a estimativa do teor de clorofilas. As extrações para obtenção do conteúdo de clorofila nas folhas da macrófita, controle e tratadas, foram realizadas a partir de 10 mg da porção mediana das folhas, a 2 cm da borda, e o teor de clorofila foi estimado pela metodologia proposta por Lichtenthaler (1987). As análises foram realizadas em todos os tempos de exposição nas plantas controle e tratadas com os metais Cu e Cr.

Pulso de Amplitude Modulada (PAM) – Método Fluorométrico

Através das análises de fluorescência foi possível estimar alguns parâmetros relacionados à atividade fisiológica e fotossintética das macrófitas expostas ao efeito do metal cromo (Cr). Os seguintes parâmetros fotossintéticos foram avaliados: "*Yield*" ou rendimento fotoquímico máximo do PSII ($\phi_M = FV_{MAX}/F_{MAX}$), rendimento fotoquímico no "steady state" do transporte de elétrons como uma medida do fotoquímica real do PSII no equilíbrio do transporte de elétrons ($\phi'_M = [Fv_S - Fv]/[Fv + F'o]$), valores do "quenching" não-fotoquímico como uma medida da dissipação da energia luminosa por uma via deferentes dos processos não-fotoquímicos ($QN = 1 - FvS/FvMAX$) e produção fotoquímica como uma medida da energia luminosa dissipada via transporte de elétrons fotossintético ($Q_P = [Fv_S - Fv]/Fv_S$) (KRAUSE; WEIS, 1991; BOLHÀR-NORDENKAMPF et al., 1989; SCHREIBER et al. 1986).

Os parâmetros fotossintéticos de ϕ_M, ϕ'_M Q_N e Q_P foram avaliados pelo uso do fluorômetro PAM (Mini PAM Walz 2007F-1, Germany) da Universidade Federal de São Carlos, cedido pelo professor Carlos Henrique B. A. Prado, do departamento de Botânica nas dependências do NEEA. As folhas de *P. stratiotes* permaneceram no escuro por 30 minutos para induzir o equilíbrio do sistema fotossintético e foram então colocadas no clipe para medição dos parâmetros fotossintéticos. As intensidades do pulso de luz saturante e actínica foram de 2000 μmol s^{-1} m^{-2} e a intensidade de luz modulada foi de 1 μmol s^{-1} m^{-2}. Cada medição foi realizada em triplicata em todos os tempos de exposição das macrófitas ao tratamento com o metal cromo (SCHREIBER et al. 1986).

Ensaios de Enzimas Antioxidantes

A atividade das seguintes enzimas foi avaliada: catalase (CAT), superóxido dismutase (SOD), glutationa redutase (GR) e ascorbato peroxidase (APX). As análises destas enzimas foram realizadas por atividade em géis não desnaturantes (poliacrilamida 8-12%) e por espectrofotometria. As extrações das enzimas foram feitas em um tampão padrão [tampão fosfato de potássio 100 mM, pH 7.0, contendo 1 mM EDTA, 1mM DTT (ditiotreitol) e 4% (p/v) PVPP (polivinilpolipirrolidona)] de acordo com Azevedo et al. (1998). O sobrenadante foi utilizado para medir as atividades enzimáticas de raiz e folha da macrófita *P. stratiotes* submetidas ao tratamento com cromo e cobre, em todos os tempos de exposição.

O conteúdo de proteínas foi avaliado de acordo com a metodologia proposta por Bradford (1976) usando a proteína soro albumina bovina como padrão.

a) Atividade da Catalase – CAT (EC 1.11.1.6)

A atividade total de CAT nas raízes e folhas de *P. stratiotes* foi determinada espectrofotometricamente por monitoramento da degradação de H_2O_2 a 240 nm por 1 min. contra uma amostra de extrato vegetal como branco conforme descrito por Azevedo et al. (1998).

A atividade de SOD e GR foi avaliada por análise eletroforética sob condições não-desnaturantes em gel de poliacrilamida 12% conforme descrito por Medici et al. (2004).

b) Atividade da Superóxido Dismutase – SOD (EC 1.15.1.1)

A atividade de SOD foi determinada como descrita por Beauchamp e Fridovich (1971) com as modificações sugeridas por Azevedo et al. (1998).

c) Atividade da Glutationa Redutase – GR (EC 1.6.4.2)

A atividade de GR em gel PAGE foi determinada como descrito por Lee e Lee (2000) com as modificações descritas por Medici et al (2004). A atividade da GR foi estimada pela redução de glutationa oxidada a qual foi acompanhada por monitoramento na alteração da absorbância a 412nm.

d) Atividade da Ascorbato Peroxidase – APX (EC 1.11.1.11)

A atividade total da APX foi avaliada nas raízes e folhas pelo método proposto por Nakano e Asada (1981), através do monitoramento da taxa de oxidação do ascorbato a 290 nm a 30 $^{\circ}$C. A atividade foi calculada usando o coeficiente de extinção de 2,8 mM cm^{-1}.

Caracterização Enzimática

No caso de isoenzimas da SOD, a classificação das mesmas ocorreu de acordo com o padrão já descrito na literatura (AZEVEDO et al., 1998), sendo a análise essencial para a pesquisa, visto que as diferentes formas desta enzima apresentam-se localizadas em organelas distintas. Existem três tipos de isoenzimas: Mn-SOD, Fe-SOD e Cu/Zn-SOD. Para a classificação das isoenzimas, os géis foram incubados em duas soluções: uma contendo 5 mM de peróxido de hidrogênio a outra contendo 2 mM KCN. Mn-SOD é caracterizada pela resistência à inibição da atividade em ambas as soluções, já a Cu/Zn-SOD é caracterizada pela sensibilidade em ambas as soluções e a Fe-SOD é inibida por peróxido de hidrogênio, mas não por KCN.

Determinação do Conteúdo de H_2O_2 e Peroxidação de Lipídios

Um dos mecanismos de exterminação das EAO (Espécies Ativas de Oxigênio) é a detoxificação dos íons superóxido catalisado pela Superóxido Dismutase (SOD), reação que produz H_2O_2 e oxigênio. Os danos induzidos pelas EAO podem ser demonstrados pela medição de malonaldeído (MDA) induzido pela peroxidação de lipídios. O conteúdo de H_2O_2

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e malonaldeído foi medido em todos os tempos de exposição ao tratamento. A peroxidação de lipídios foi avaliada através da produção de metabólitos reativos a ácido 2-tiobarbitúrico (TBA), principalmente malondialdeído (MDA) (HEATH; PACKER, 1968). Os resultados foram expressos em conteúdo de malondialdeído (MDA), usando um coeficiente de extinção de 155 mM cm^{-1}.

3.1.3. Remoção de Nutrientes por Macrófitas em "*Wetlands*" Construídos sob Diferentes Regimes de Fluxo.

A terceira etapa da pesquisa foi realizada durante o estágio de doutorado sanduíche, no Departamento de Biodiversidade e Gestão Ambiental, da Faculdade de Ciências Biológicas e Ambientais da Universidade de León, na Espanha, e para melhor entendimento desta etapa um esquema é apresentado na figura 2.

O trabalho desenvolvido neste estágio visou: comparar a capacidade de remoção de amônia e fosfato nos wetlands submetidos a dois regimes de fluxo, superficial e subsuperficial; comparar a remoção de nutrientes associada às mudanças no volume efetivo de água nos *wetlands* e no tempo de retenção hidráulica; e também comparar a capacidade de remoção associada à presença das macrófitas: *Typha* sp. e *Phragmites* sp., ou ausência das macrófitas. A tabela 5 apresenta todas as comparações propostas entre os oito wetlands apresentados na figura 2.

Tabela 5. Comparações realizadas entre os wetlands construídos (WC) visando atender os objetivos da terceira etapa da pesquisa.

WC comparados	Efeitos comparados:
1 x 5	Capacidade dos WC na remoção de nutrientes plantados com ambas as macrófitas
2 x 3	Capacidade dos WC sob diferentes regimes de fluxo
3 x 4	Capacidade dos WC na remoção de nutrientes, com e sem a presença da macrófita *Typha* sp.
6 x 7	Capacidade dos WC na remoção sob efeito de carga diferencial
7 x 8	Capacidade dos WC na remoção de nutrientes, com e sem a presença da macrófita *Phragmites* sp.
4 x 8	Teor de nutrientes entre os WC sem vegetação
3 x 7	Capacidade dos WC na remoção de nutrientes plantados com ambas as macrófitas, e com diferenças volume efetivo de água e tempo de retenção hidráulica
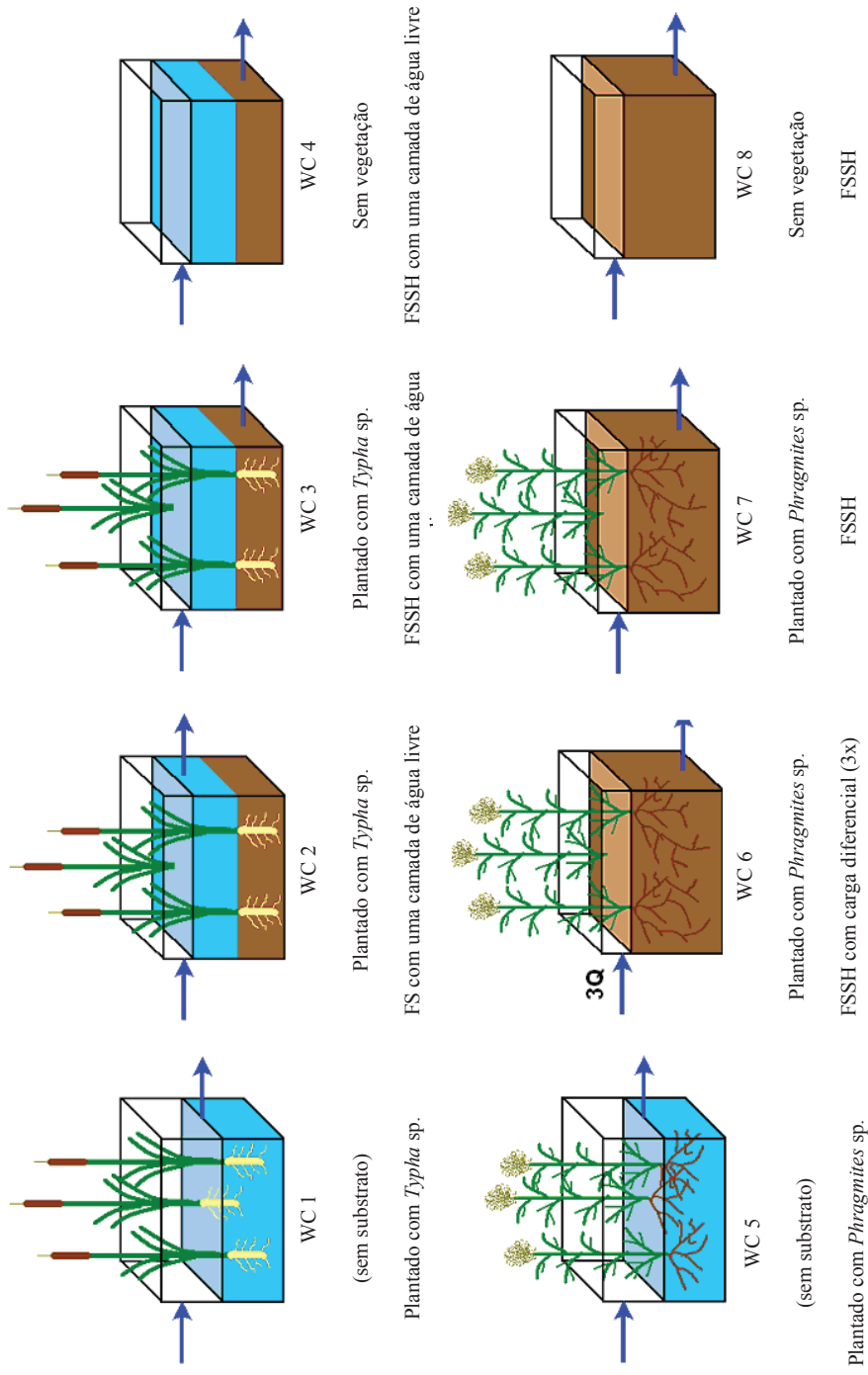
Figura 2. Representação esquemática da estrutura experimental dos *wetlands* construídos (WC) com e sem o cultivo de macrófitas (*Typha* sp. e *Phragmites* sp.) sob diferentes regimes de fluxo. (FS, fluxo superficial; FSSH, fluxo subsuperficial horizontal).

WC 1
(sem substrato)
Plantado com *Typha* sp.

WC 2
Plantado com *Typha* sp.
FS com uma camada de água livre

WC 3
Plantado com *Typha* sp.
FSSH com uma camada de água

WC 4
Sem vegetação
FSSH com uma camada de água livre

WC 5
(sem substrato)
Plantado com *Phragmites* sp.

WC 6
Plantado com *Phragmites* sp.
FSSH com carga diferencial (3x)

WC 7
Plantado com *Phragmites* sp.
FSSH

WC 8
Sem vegetação
FSSH

Para avaliar e comparar a *capacidade* dos oito wetlands foram analisados os seguintes parâmetros: pH, concentração de oxigênio dissolvido (OD) e potencial redox. Os teores de amônia e fosfato dos WC foram comparados em relação à concentração de entrada do efluente doméstico aplicado, em porcentagem de remoção e em taxa de carregamento (g m^{-2} dia^{-1}). Os dados utilizados nas comparações foram referentes ao período de junho/2007 a fevereiro/2008 (verão/2007 e inverno/2008) e de julho a setembro de 2008 (verão/2008). Os referidos dados foram submetidos à análise de variância (ANOVA) pelo programa Statistic 7.0 software (Statsoft, USA) e as diferenças entre as medias avaliadas pelo teste-*t*.

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CAPÍTULO 4. A STUDY OF THE EFFECTS OF CHROMIUM EXPOSURE ON THE GROWTH OF *PSEUDOKIRCHNERIELLA SUBCAPITATA* (KORSHIKOV) HINDAK EVALUATED BY CENTRAL COMPOSITE DESIGN (CCD) AND RESPONSE SURFACE METHODOLOGY (RSM)

ABSTRACT

The aim of this study was to evaluate the effects of chromium exposure on the growth of *P. subcapitata* using the Central Composite Design (CCD) and Response Surface Methodology (RSM). The highest values for algal density and biomass were obtained in the longest exposure times and for the lowest chromium concentrations. The CCD used for the analysis of treatment combinations showed that a second order polynomial regression model was in good agreement with experimental results, with $R^2 = 81.50$ and 89.90; for algal density and biomass ($p < 0.05$), respectively. Only the exposure time was significant for algal density. For chlorophyll, in contrast, the exposure time, chromium concentration and their interaction significantly affected the growth of *P. subcapitata*. The findings confirmed the sensitivity of *P. subcapitata* to chromium (VI), which makes it a suitable bioindicator of environmental contamination for this metal.

Keywords: algae; CCD; metal; RSM; *Selenastrum capricornutum*; toxicity.

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Estudo dos Efeitos da Exposiçao do Cromo no Crescimento de *Pseudokirchneriella subcapitata* (Korshikov) Hindak avaliado pelo Delineamento Composto Central (DCC) e Metodologia de Superfície de Resposta

O objetivo deste estudo foi avaliar o efeito da exposição ao cromo no crescimento de *P. subcapitata* utilizando o Delineamento Composto Central (DCC) e a Metodologia de Superfície de Resposta (MSR). Os maiores valores de densidade algal e biomassa foram obtidos nos maiores tempos de exposição e nas concentrações mais baixas de cromo. O DCC foi utilizado para analisar as combinações de tratamento mostrando que um modelo de regressão polinomial de segunda ordem apresentou um bom ajuste com os resultados experimentais, com R2 = 81,50 e 89,90, para densidade e biomassa algal ($p<0,05$), respectivamente. Apenas o tempo de exposição foi significante para a densidade algal. Para a clorofila, por outro lado, o tempo de exposição, a concentração de cromo e a interação entre ambos afetaram significativamente o crescimento de *P. subcapitata*. Os resultados confirmam a sensibilidade de *P. subcapitata* ao cromo (VI) os quais nos permite indicá-la como bioindicadora de poluição ambiental para este metal.

Palavras-chave: algas; DCC; metais; MSR; *Selenastrum capricornutum*; toxicidade.

Environmental contamination with heavy metals may cause direct and/or indirect effects on terrestrial and aquatic ecosystems (FLEEGER et al., 2003). The toxicity of a substance (e.g. metals) to algae is usually assessed by standard growth inhibition tests using conventional species (PEREIRA et al., 2005). The most frequently used freshwater alga is *Selenastrum capricornutum* Printz, renamed *Pseudokirchneriella subcapitata* (Printz) Korshikov 1990 (PARDOS et al., 1998), and studies have mostly focused on measuring endpoints resulting from chronic exposures of usually 3-4 days (LEWIS, 1995).

In traditional experiments, most test designs are generated in such a way that one parameter is varied while the others are kept constant. The disadvantage of this type of design is twofold: the effect of only one parameter is studied, and possible interactions of different parameters cannot be assessed (HEIJERICK et al., 2003). In contrast, the advantage of the Central Composite Design (CCD) is that it can generate a maximum amount of information on the direct effect of test variables and their interactions while testing a minimum number of combinations (DE SCHAMPHELAERE et al., 2003).

Thus, a way to develop, improve and optimize processes consists of applying the CCD and the Response Surface Methodology (RSM), which can be very useful and advantageous for both the evaluation and optimization of some capacidade parameters. More specifically, the experimental design helps the researcher to verify if changes in the independent variables produce a statistically significant variation of the observed response, and this approach can be used each time this type of information is required (FURLANETTO et al., 2003).

The aim of this study was to evaluate the effects of chromium concentration and exposure time on the growth of *P. subcapitata* using the CCD and the RSM analysis. This experimental design generated mathematical models to predict chromium (Cr^{+6}) toxicity in *Pseudokirchneriella subcapitata.* The second-order polynomial model was used in the simulation to provide a better understanding of the effect of chromium concentration and exposure time on algal growth.

4.2. MATERIAL AND METHODS

Algal culture

The green algae *Pseudokirchneriella subcapitata* were obtained from cultures maintained at the Ecophysiology Laboratory for Aquatic Organisms, Center of Water Resources and Applied Ecology (CRHEA), University São Paulo, and were cultivated in L.C. Oligo medium (AFNOR 1980), which was first autoclaved (121°C) for 15 min in 2-L Erlenmeyer flasks containing one liter of the medium (ABNT 2005). The composition of the synthetic culture medium per liter was the following: 0.17 M $Ca(NO_3)_2.4H_2O$, 0.99 M KNO_3, 0.12 M $MgSO_4.7H_2O$, 0.29 M KH_2PO_4, 0.00012 M $CuSO_4.5H_2O$, 0.00009 M $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.0002 M $ZnSO_4.7H_2O$, 0.0001 $CoCl_2.6H_2O$, 0.0002 M $Mn(NO_3)_2.4H_2O$, 0.0005 M $C_6H_8O_2.H_2O$, 0.001 M H_3BO_3, 0.005 M $C_6H_5FeO_7.5H_2O$, 0.002 M $FeSO_4.7H_2O$, 0.002 M $FeCl_3.6H_2O$, 0.2 M $NaHCO_3$.

An algal inoculum was prepared for each sample from fresh culture stocks sampled during the exponential growth phase, and the culture was kept at a temperature of 23±2°C and under a constant irradiance of 1500 lux, provided by cool-white fluorescent lamp and constant aeration (ABNT, 2005).

Toxicity tests

Tests were performed in 250 mL Erlenmeyer flasks with 100 ml of test medium and cells of *P. subcapitata* in the exponential growth phase. At the beginning of each test, each flask was inoculated with a concentration of approximately 10^4 cells ml^{-1} (ABNT, 2005). The chromium ($K_2Cr_2O_7$) concentrations were 40.0, 41.5, 45.0, 48.5 and 50.0 µg L^{-1}, and the exposure times ranged from 81 to 183 h. Static toxicity tests were conducted in the same conditions described above for the algal culture maintenance procedure. Initial and final densities were verified by cell counts in an Improved Neubauer Bright-Line hemocytometer under an optical microscope (Carl Zeiss), standard model 25. All the aliquots were counts in triplicates. The mean number of cells produced at each concentration, after the exposure period, was expressed as a percentual growth reduction with respect to the control (RODGHER; ESPÍNDOLA, 2008). These percentages were used to calculate the IC50 chromium value (effective metal concentration causing 50% inhibition of algal growth after 96 h exposure) for the algae was determined by the Trimmed Spearman-Karber method (HAMILTON et al., 1977).

Algal Biomass

The algal biomass was estimated from analysis of chlorophyll *a*. Samples (5-10 mL) from each test flask were filtered through a 0.45µm membrane filter. Boiling ethanol (80%) was poured over the filter into a beaker and, after a few minutes of cooling, the filter was grinded with mortar and pestle to facilitate extraction. The filter slurry was rinsed with ethanol and passed through a hard paper filter into a calibrated tube. The extraction process was performed in the dark for 6 to 24 hours. Measurements were at 665 nm and 750 nm in a

spectrophotometer (0.2 to 0.4 mm slit width) (F600, FEMTO, USA) against a reference cuvette filled with 80% ethanol (NUSCH, 1980). All samples were analyzed in triplicates.

Experimental Design and Statistical Analysis

Algal toxicity tests were conducted in 10 experiments for the study of two parameters (see matrix in table 1). The model studied is a 2^2 experimental design, where selected time of exposure (X1) and chromium concentration (X2) were treated as independent variables that affected density and algal biomass. Each of the parameters was coded at five levels: -1.41, -1, 0, 1, and 1.41. The range and levels of the variables in this study are shown in Table 1. RSM consists of a group of empirical techniques devoted to the evaluation of relationships existing between a cluster of controlled experimental factors and the measured responses according to one or more selected criteria (BAYRAKTAR, 2001). According to this design, the total number of treatment combinations was $2^k + 2k + n_0$, where 'k' is the number of independent variables and 'n_0' is the number of repetitions of the experiments at the center point. Based on the parameter estimates, the application of RSM provided an empirical relationship between the response variable and the test variables (QIAO et al., 2009). By performing multiple regression analyses of the experimental data, the predicted response Y for density and algal biomass can be obtained through the second-order polynomial equation:

$$Y = b_0 + b_1\,x_1 + b_2\,x_2 + b_{11}\,x_1^{\,2} + b_{22}\,x_2^{\,2} + b_{12}\,x_1\,x_2 + \varepsilon \quad (1)$$

b_0, b_1, b_2, b_{11}, b_{22}, b_{12} are constant coefficients, and x_1, x_2 are the coded independent variables or factors. The test factors were coded according to the following regression equation:

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$$x_i = \left(\frac{X_i - X_0}{\Delta X_i} \right) \qquad\qquad (2)$$

where x_i is the *coded* value and X_i is the *actual* value of the ith independent variable, X_0 is the actual value at the center point, and ΔX_i is the step change value. In this case, $X_1 =$ (time - 132)/36; $X_2 =$ ([Cr] − 45.0)/3.5 were used. The linear, quadratic and interactive effects of parameters on metal toxicities were analyzed with Statistica 7.0 software (Statsoft, USA). In order to develop a mathematical prediction model, a backward regression analysis ($p < 0.05$) was also applied to the toxicity data of exposure time and Cr (VI). Based on this parameter estimate, the model can be statistically validated if it is able to reproduce the observed behavior (FALLER et al., 2003).

Table 1. Process variables used in the CCD showing the treatment combinations between chromium concentration and exposure time.

| Treatment | Coded setting levels | | Actual levels | |
| | x1= time; x2= [Cr] | | X1= time (h); X2= [Cr] (μg L^{-1}) | |
	x1	x2	X1	X2
1	-1	-1	96	41.5
2	-1	1	96	48.5
3	1	- 1	168	41.5
4	1	1	168	48.5
5	0	0	132	45.0
6	0	0	132	45.0
7	-1.41	0	81	45.0
8	0	- 1.41	132	40.0
9	1.41	0	183	45.0
10	0	1.41	132	50.0

CCD was used to determinate the toxic effect of chromium on algal density and chlorophyll *a* concentration or algal biomass. Analysis of variance (ANOVA) was employed to determine significant parameters and to estimate algal density and biomass as a function of exposure time and chromium concentration. The quality of fit of the second-order model

equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by an F-test (ANOVA). The significance of the regression coefficients was tested by a t-test.

4.3. RESULTS

Table 2 shows the results of the experimental design for density and algal biomass that was used to investigate the influence of chromium on *P. subcapitata* for different exposure times.

Table 2. The mean experimental and standard errors in parentheses from design responses with the results obtained from algal density and algal biomass.

Treatment	Algal Density $(10^4 \text{ cells mL}^{-1})$	Biomass $(\mu g/l)$
1	143 (8)	78.1 (7)
2	57 (2)	136.7 (16)
3	776 (44)	505.0 (73)
4	78 (9)	20.9 (1)
5	489 (15)	263.7 (15)
6	404 (9)	284.6 (25)
7	71 (2)	9.8 (0)
8	176 (9)	443.6 (51)
9	741 (42)	502.2 (52)
10	267 (22)	132.5 (10)

The results suggest that both algal density (Figure 1) and algal biomass (Figure 2) presented the smallest growth under the longest exposure time and the highest chromium concentration. The longest exposure time and lowest chromium concentration provided the best conditions for algal growth (Figure 1).

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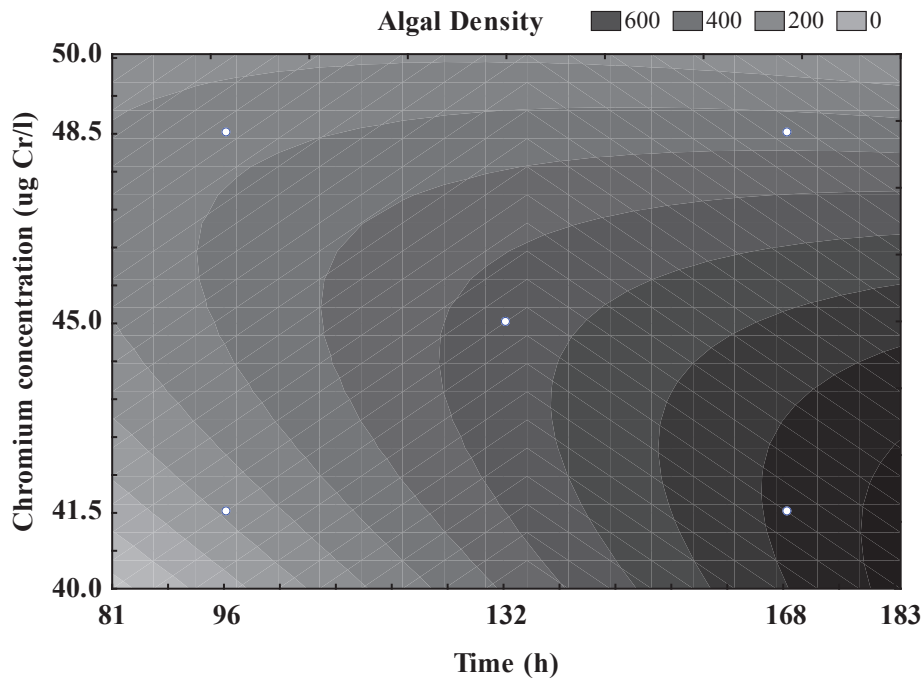
Figure 1. Contour plot of algal density as a function of chromium concentration (40.0-50.0 µg L^{-1}) and exposure time (81-183 h) for *P.subcapitata*

Contour plot indicates that maximum density and algal biomass were attained for the longest exposure time range (168-183 h). However, the ranges of chromium concentration were narrower 40-41.3 µg Cr L^{-1} for algal biomass, than 40-44.3 µg Cr L^{-1} for algal density.
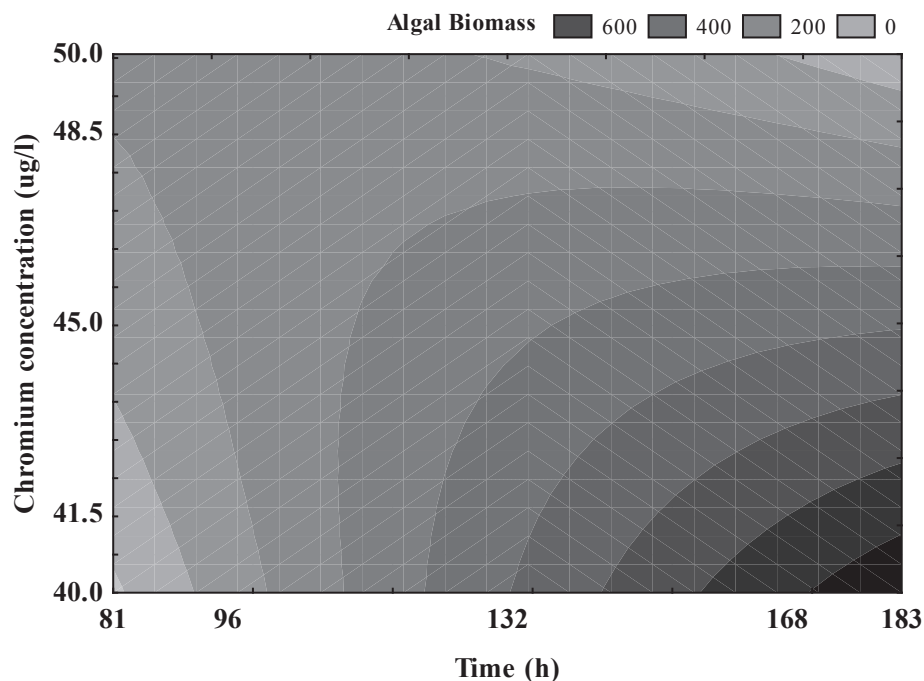
Figure 2. Response surface of algal biomass as a function of chromium concentration (40.0-50.0 μg L^{-1}) and exposure time (81-183 h) for *P.subcapitata.*

The first step in the design is therefore to take the declared objective of the experiment and translate it into some quantitative measurement that can be estimated, such as an EC50 (the "effective concentration", or concentration of test chemical that affects 50% of the organisms tested) (CHAPMAN et al., 1996). The half maximal inhibitory concentration (IC50) was estimated in order to assess the sensitivity of *P. subcapitata* to different chromium concentrations in different exposure times. In addition, based on the percentages of growth reduction and IC50 values obtained by the Trimmed Spearman-Karber method, the CCD coded models made it possible to generate data simulation to obtain IC50 values for different exposure times. Thus, it was possible to calculate IC50 at 96, 132, 168 and 183h with 95% reliability. The values obtained were: 42.78 and 43.54 (limits not defined), 47.58 (47.26-47.90) and 45.62 (44.88-46.37) μg Cr L^{-1}, respectively.

A model fitting was performed for the experimental design, and the ANOVA (Analysis of Variance) was used to evaluate the adequacy of the fitted model. Table 3 shows the resulting model coefficients estimated by regression analysis. These findings suggest that the models were significant, and the coefficient of determination estimate indicate that 81.5 %, for algal density, and 89.9 %, for algal biomass, of the variability in these responses could be accounted for by the model, which is indicative that the model provides adequate representation.

Table 3. Obtained model and regression coefficients to Equation (1) and analysis of variance (ANOVA) for the experiments.

Term	Algal Density (10^4 cells/ml)		Biomass (μg/l)	
	Coefficients and Standard Error ($\pm$)	p value	Coefficients and Standard Error ($\pm$)	p value
Mean/Interc b_0	*446.50 (124.12)*	0.02	*279.35 (64.92)*	0.01
t (L) b_1	*200.13 (62.06)*	0.03	*125.94 (32.46)*	0.02
t (Q) b_2	-32.67 (82.10)	0.71	-33.39 (42.94)	0.48
c (L) b_{11}	-82.03 (62.06)	0.26	*-108.18 (32.46)*	0.03
c (Q) b_{22}	-125.00 (82.10)	0.20	-17.35 (42.94)	0.71
t*c b_{12}	-153.00 (87.77)	0.16	*-135.66 (45.91)*	0.04
R^2	81.50	-	89.90	-
R^2 adjusted	58.30	-	77.20	-
F-value	8.79	-	17.75	-
Df	1/9	-	3/9	-
F cal/Ftab	1.72	-	4.60	-

The values in bold and italic are significant $p < 0.05$, with confidence level 95%.

There was significance for coefficients determined by Student's t-test (Table 3). In this study, only the mean and the linear time were significant (p time-$t < 0.05$) for algal density. Considering the second-order model of algal biomass, various coefficients were significant: the mean, the time and linear chromium concentration, and the interaction between them ($p <$

0.05). These findings indicate that they can act as limiting factors and their values might change responses in algal density and algal biomass to a considerable extent.

In addition, the CCD coded models permitted data simulation to IC50. The generated models were used to run the simulations of density and algal biomass (Figure 3a and b).
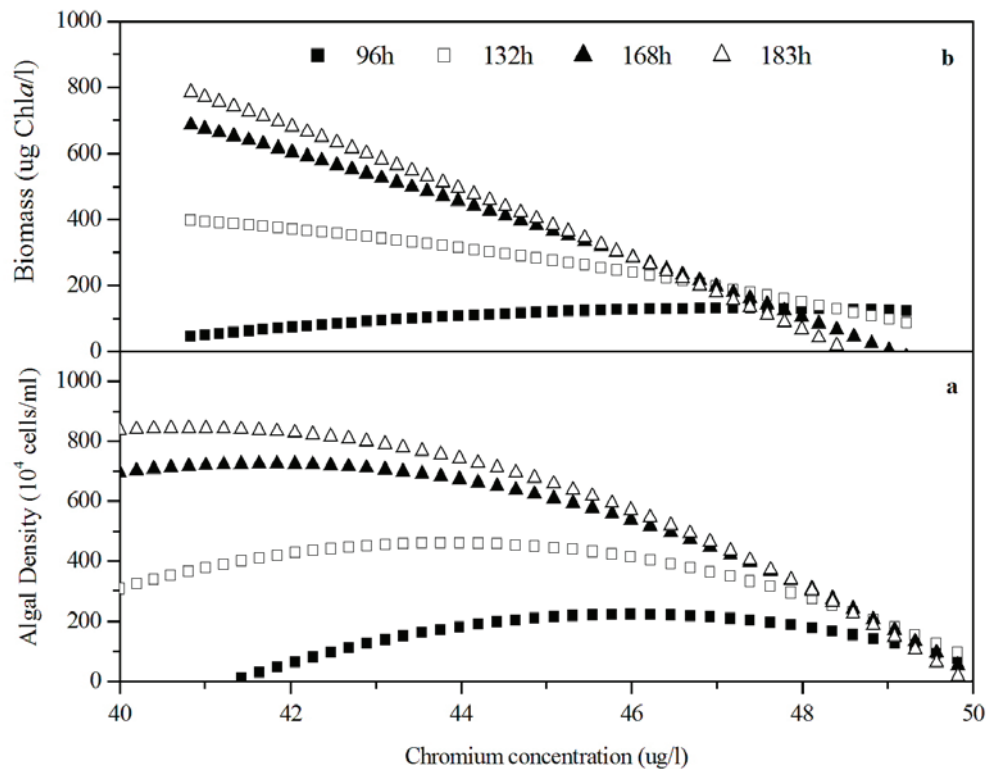
Figure 3. Simulation of algal density (a) and algal biomass (b), as a function of chromium concentration (40.0-50.0 µg L^{-1}) in different exposure time for *P.subcapitata*. ■: 96h; □: 132h; ▲: 168h; ∆: 183h.

The data simulation works similarly for algal density and biomass. At the initial exposure time (96h) there is a slight, proportional increase as the chromium concentration rises, and later there is a drop. The difference between the parameters is the chromium concentration at which the curve inflection occurs: 46.0 µg Cr L^{-1} for algal density and 48.0

µg Cr L^{-1} for biomass. For the subsequent exposure times (132 a 183 h), there was a reduction in algal biomass while chromium concentration increased.

4.4. DISCUSSION

Consistent algal growth inhibition was observed in this study, especially for the highest chromium concentrations and the longest exposure time (Figures 1 and 2). However, Labra et al. (2007) and Pereira et al. (2005) also observed growth inhibition, though for a range of very low chromium concentrations (1.0-7.5 µg Cr L^{-1}) and soon after 24h of treatment.

Rodgher et al. (2008) studied algal cells of *P. subcapitata* as routes for copper exposure and toxicity to cladocerans. Reductions were found in algal density and in chlorophyll *a* content for the algae subjected to the treatment with copper. The correlation analysis between algal cell densities and chlorophyll *a* content confirmed that chlorophyll-a reduction was a function of reduced algal cell density. These results are consistent with our data and can be also visualized on data simulation (Figure 3).

A decrease in total chlorophyll, chlorophyll *a* and *b*, and carotenoids has been well documented in plants, moss and algae under Cr stress (CHOUDHURY; PANDA, 2005; PANDA, 2003; PANDA et al., 2003; PANDA; CHOUDHURY, 2005). Experiments performed with *P. subcapitata* subjected to higher copper concentrations (1.0 and 1.5 mg L^{-1}) induced chlorophyll degradation (CVETKOVIC et al., 1991; RODGHER et al., 2008).

Furthermore, the IC50 (half maximal inhibitory concentration) is a measurement of the effectiveness of a compound in inhibiting biological or biochemical functions. Although most standard algal assays used for regulatory purposes appear to have similar designs and operating procedures, subtle differences in test design may lead to a large variability in

results. Nevertheless, it is unclear whether the differences in sensitivity among various algal taxas and within individual species reported in the current literature are caused by differences among the various biotic and abiotic factors in standard operating procedures (JANSSEN; HEIJERICK, 2003). Such variability can be verified in data obtained by different authors (MASUTTI, 2004; RODGHER; ESPÍNDOLA, 2008; ROJÍCKOVÁ; MARŠÁLEK, 1999; TURBAK et al., 1986) (Table 4).

Table 4. Different IC50 values of chromium (VI) reported in the literature for *P. subcapitata*.

IC50	$[Cr^{+6}]$	Exposure time	Reference
542.71	$\mu g\ L^{-1}$	96 hours	(RODGHER; ESPÍNDOLA, 2008)
420.0	$\mu g\ L^{-1}$	96 hours	(MASUTTI, 2004)
396.1	$\mu g\ L^{-1}$	96 hours	(ROJÍČKOVÁ; MARŠÁLEK 1999)
238.0	$\mu g\ L^{-1}$	96 hours	(TURBAK et al., 1986)
42.78	$\mu g\ L^{-1}$	96 hours	In this work

The reduction in viable cell numbers was observed by Labra et al. (2007) and Pereira et al. (2005), suggesting that potassium dichromate is a strong algal cell pollutant and *P. subcapitata* is a suitable sensitive organism to monitor the presence of chromium in water. In addition, a direct relationship between Cr content and cell mortality was found only when the amount of Cr was related to protein content in *Scenedesmus acutus* treated with Cr (VI) (GORBI et al., 2001). Corradi et al. (1998) suggested that the ability of *S. acutus* to detoxify chromium was related to the higher production of carbohydrates and proteins in response to metal exposure.

In another study, the higher tolerance of *Chlorella kessleri* was accounted for by differences in production of extracellular organic substances under stressful conditions in comparison with *P. subcapitata* and *Scenedesmus quadricauda* (*P. subcapitata* was considered the most sensitive) (MARŠÁLEK; ROJÍČKOVÁ, 1996).

Considering that *P. subcapitata* responses to contaminants, such as heavy metals, are typically measured in terms of biomass, cell density, growth rate, etc (LABRA et al., 2007), the use of bioassays provides a direct and integrated estimate of the heavy metal's toxicity. Recently, environmental agencies have focused on optimizing methods, endpoints and test organism selection. Statistical advice in current ecotoxicity test guidelines is in need of improvement. More advice should be given on experimental design, statistical analysis and ways of reporting results (CHAPMAN et al., 1996).

4.5. CONCLUSION

The results from this study confirmed the sensitivity of *P. subcapitata* to chromium (VI), which makes it a suitable bioindicator of environmental contamination by this metal. The Central Composite Design represents a valuable tool to determine mathematical relationships to predict toxicity, which allows the simulation of any response (dependent variables) around a range of tested factors (independent variables). The use of CCD in aquatic environmental toxicology is a powerful technique for investigating multivariate systems (because many factors may interact simultaneously in the environment). It reduces the number of experiments and repetitions without loss of statistical reliability (since it is possible to calculate the experimental error). Besides, it increases the predictions and efficiency of data sets and reduces experimental residual volume.

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CAPÍTULO 5. ASSESSMENT OF CHROMIUM BIOACCUMULATION IN *PSEUDOKIRCHNERIELLA SUBCAPITATA* (KORSHIKOV) HINDAK BY THE CENTRAL COMPOSITE DESIGN (CCD) AND RESPONSE SURFACE METHODOLOGY (RSM).

ABSTRACT

The effects of chromium bioaccumulation in *Pseudokirchneriella subcapitata* were evaluated by Central Composite Design (CCD), factorial 2^2 and Response Surface Methodology (RSM). All the models of regression generated by CCD were highly significant, with R^2 between 77 and 88%, which is the percentual variability in the response that the model can account for. This is indicative of a satisfactory representation of the process model whose data can be used for simulations of response. The maximum shrinkage biovolume presented 28–69% reduction compared to controls. Results from this study suggest that the smaller algal cells amplify metal binding sites, leading to an increased bioaccumulation and a consequential increased capacity to accumulate chromium. Nevertheless, the absorption capacity decreases for more elevated chromium concentrations and for longer exposure.

Keywords: Algae, Biovolume, Central Composite Design, Metal, *Selenastrum capricornutum*.

RESUMO

Avaliação da bioacumulação de cromo em *Pseudokirchneriella subcapitata* (Korshikov) Hindak pelo Delineamento Composto Central (DCC) e Metodologia de Superfície de Resposta (MSR)

Os efeitos da bioacumulação de cromo em *Pseudokirchneriella subcapitata* foram avaliados pelo Delineamento Composto Central (DCC), fatorial 2^2 e pela Metodologia de Superfície de Resposta (MSR). Todos os modelos de regressão gerados pelo planejamento experimental foram altamente significativos com R^2 entre 77 e 88%, o qual representa o percentual de variabilidade da resposta que pode ser explicado pelo modelo. Isto é indicativo de uma representatividade satisfatória do modelo gerado, cujos dados podem utilizados para a simulação de respostas. A faixa de redução do biovolume foi de 28-69% em comparação com o controle. Os resultados deste estudo sugerem que células menores amplificam sua área de superfície e os sítios de ligação com os metais, conduzindo a um aumento da bioacumulação e um conseqüente aumento da capacidade de retenção do cromo. Apesar disso, a capacidade de bioacumulação decresce nas concentrações mais elevadas de cromo e nos maiores tempos de exposição.

Palavras-chave: algas, biovolume, metais, planejamento experimental, *Selenastrum capricornutum*.

Water contamination with heavy metals is currently a very serious problem in the world (MAINE et al., 2004; VENAY et al., 2007) and represents an important environmental concern due to the toxic effects of metals, and their accumulation throughout food chains leads to serious ecological and health hazards (MALIK, 2004). Chromium is a highly toxic non-essential metal for microorganisms and plants (CERVANTES et al., 2001).

The interest in chromium (Cr) originates from the widespread use of this metal in various types of industries, such as the metallurgical (steel, iron- and nonferrous alloys), refractory (chrome and chrome-magnesite), and chemical (pigments, electroplating, tanning and other) segments. As a result of industrial processes, large amounts of Cr compounds are discharged into the environment as liquid, solid, and gaseous wastes which can ultimately cause significant adverse biological and ecological effects (KOTAŚ; STASICKA, 2000). In nature, chromium (Cr) exists in two different oxidation states: trivalent (Cr III) and hexavalent (Cr VI) chromium (KOTAŚ; STASICKA, 2000; PANDA; CHOUDHURY, 2005), the most stable and most usual forms of the chemical (CERVANTES et al., 2001). Both Cr (III) and Cr (VI) differ in terms of mobility, bioavailability and toxicity. The hexavalent form of the metal, Cr (VI), is considered a more toxic substance than the relatively innocuous and less mobile Cr (III) form (KOTAŚ; STASICKA, 2000; CERVANTES et al., 2001; PANDA; CHOUDHURY, 2005).

Bioavailability and bioaccumulation of heavy metals in aquatic ecosystems are gaining tremendous significance globally (VARDANYAN; INGOLE, 2006). Land plants, aquaticplants and algae have all drawn considerable attention for their capabilities to eliminate heavy metals. Macro and microalgae exhibit constitutive mechanisms for the

removal of free metal ions from water, which makes them attractive agents in both water detoxication and remediation processes (PERALES-VELA et al., 2006). Algae meet all the basic requirements for bioindicators: they are sedentary, their dimensions are suitable, they are easy to identify and to collect, they are widely distributed, and they accumulate metals to a satisfactory level (CONTI et al., 2002). The freshwater alga *Pseudokirchneriella subcapitata* (Korshikov) Hindak, 1990 has become the mainstay in biomonitoring and for evaluating the toxicity of chemicals and wastewater (WARD et al., 2002). The response of *P. subcapitata* to contaminant exposure, such as heavy metals, is typically measured in terms of biomass, cell density, growth rate, etc (LABRA et al., 2007).

Recently, the need and importance have been acknowledged of developing validation procedures for models that make it possible to monitor the environmental quality of aquatic ecosystems. The Response Surface Methodology (RSM) and the use of experimental design or the Central Composite Design (CCD) represent the use of techniques that warrant traceability, support validation and produce the subsequent confirmatory validation, in addition to making it possible to understand the effect of several variables on a system by means of a well-defined mathematical model. In particular, statistical design is criterion for choosing experiments efficiently and systematically in order to generate reliable and consistent information (FURLANETTO et al., 2003). The experimental design generates a mathematical model in which the parameters are estimated from experimental data. These data are also required for the simulation to occur. This technique is widely used due to its flexibility, simplicity and realism (KLEIJNEN; STANDRIDGE, 1988).

The objective of this study was evaluated the bioaccumulation of chromium in *P. subcapitata* through to CCD and the use of RSM. The experiments were conducted using algal culture subjected to different chromium concentrations for different exposure times. The metal accumulated by algal cells, and the ratio between chromium content and biovolume

were analyzed. An experimental simulation data set was used in order to improve the comprehension of the effect of chromium concentration and exposure time on bioaccumulation and biovolume variation.

5.2. MATERIAL AND METHODS

Algal culture

The freshwater green algae *Pseudokirchneriella subcapitata*, formerly known as *Selenastrum capricornutum* Pintz, (cultures kept at the Ecotoxicology and Ecophysiology Laboratory for Water Organisms of the Water Resources and Applied Ecology Center of the University of São Paulo, São Paulo State, Brazil) was cultivated in L. C. Oligo medium that does not contain ethylenediaminetetraacetic acid (EDTA) (AFNOR, 1980). Culture media were sterilized by autoclaving at 121 °C during 15 minutes in 2-L glass Erlenmeyer flasks containing 1L of the culture medium (ABNT, 2005). The cultures were maintained under continuous cool-white fluorescent lighting (1500 lux) with 12:12 PM light/dark cycle, at 24 ± 2 °C, with constant aeration and was inoculated with cells to a concentration around 1×10^4 cell.mL^{-1}. For each sample, an algal inoculum was prepared from fresh culture stocks sampled during the exponential growth phase, and the culture was maintained at 23 ± 2°C and under a constant irradiance of 1500 lux, provided by cool-white fluorescent lamp and constant aeration (ABNT, 2005).

Toxicity tests

Glass Erlenmeyers flasks (250 mL) with 100 mL of test medium, were inoculated at an initial cell density corresponding to the beginning of logarithmic phase growth with concentration around the 10^4 cells mL^{-1}. The test solutions were prepared using glass flasks and volumetric pipettes with nominal chromium concentrations (K$_2$Cr$_2$O$_7$) of 40.0, 41.5, 45.0, 48.5 and 50.0 μg L^{-1}, diluted with L. C. Oligo medium. After inoculation, static toxicity tests were maintained for exposures time: 81, 96, 132, 168 and 183h in the same conditions described above for the algal culture maintenance procedure. The chromium concentrations and exposures time were obtained by combinations according to experimental design. The Erlenmeyers were repositioned daily to minimize possible spatial differences in illumination and temperature on growth.

Algal biovolume

The algal biovolume was calculated by means of measured linear dimensions that include the full range of microalgal shapes and mathematical equations (HILLEBRAND et al., 1999). The measurements required in order to calculate the mean biovolume were taken from 30 specimens (or cells) for each run, and measurements in .

Metal analysis

At the end of the each exposure time, samples with *P. subcapitata* were sampled to determine the bioaccumulation, expressed as the metal accumulated by algal cells (including the metal absorbed by cells and the metal bound externally). The trial test solutions were filtered through a membrane filter AP20 (Milipore) with 0.45 μm. The filters were dried and submitted to acid digestion (HNO$_3$ and H$_2$O$_2$) (APHA, 1995). For each digested sample, three

unused filters were digested and analyzed as blanks (VAN LOON, 1985). Generally, the measured concentration of metal in algal cells is taken as the total amount of metal accumulated by the cells (i.e., externally and internally bound metal) and it is expressed as mg Cr mg^{-1} dry weight of algae or µg Cr cell^{-1}. All the samples were analyzed in triplicates by graphite-furnace atomic absorption spectrometry (Varian AA 220). The data for metal concentration were used to calculate the ratio to the biovolume, expressed as µg Cr µm^{-3}.

Experimental Design and Statistical Analysis

Central composite design (CCD) was used in order to generate 10 treatment combinations ($k = 2$) for the selected algal toxicity tests is that in which two parameters (exposure time (X1) and chromium concentration (X2) as independent variables. Five levels of each variable were chosen, the upper and lower limits of them, relative to the opted center point (exposure time 132h and chromium concentration 45.0 µg L^{-1}). The experimental results for the response surface methodology were fitted with a second-order polynomial equation (1) by a multiple regression technique.

$$Y = b_0 + b_1\ x_1 + b_2\ x_2 + b_{11}\ x_1^{\ 2} + b_{22}\ x_2^{\ 2} + b_{12}\ x_1\ x_2 + \varepsilon \quad (1)$$

Y is the predicted response; b$_0$, b$_1$, b$_2$, b$_{11}$, b$_{22}$, b$_{12}$ are constant coefficients, and x_i is the coded value and X_i is the actual value of the ith independent variable, X_0 is the actual value at the center point, and ΔX_i is the step change value. Statistic 7.0 software (Statsoft, USA) was used for regression and graphical analysis of the data. The significance of the regression coefficients was determined by Student's t-test; the second order model equation was determined by Fisher's test. The variance explained by the model is given by the multiple

coefficient of determination, R^2. Based on this parameter estimate, the model can be statistically validated if it is able to reproduce the observed behavior (FALLER et al., 2003).

The test factors were coded according to the following regression equation (2):

$$x_i = \left(\frac{X_i - X_0}{\Delta X_i} \right)$$

(2)

where x_i is the coded value and X_i is the actual value of its independent variable, X_0 is the actual value at the center point, and ΔX_i is the step change value. In this case, X_1 = (time - 132)/36; X_2 = ([Cr]–45.0)/3.5 were used.

5.3. RESULTS AND DISCUSSION

Effect of chromium bioaccumulation in *P. subcapitata*

Table 1 shows the actual levels corresponding to the coded settings, the treatment combinations and responses. The bioaccumulation of metal in *P. subcapitata* was evaluated following the analysis of metal accumulated by algal cells at different exposure times and chromium concentrations (Table 1).

Table 1. Process variables used in the CCD showing the treatment combinations between chromium concentration and exposure time and the mean experimental responses obtained for bioaccumulation, biovolume and the ratio between both, as well as the percentual biovolume reduction in relation to the control.

Treatment	Coded setting levels		Actual levels		Bio-acculumation (μgCr gDW^{-1})	Bio-volume (μm^3)	Per cent biovolume reduction	Ratio [Cr] per biov (μg μm^{-3}x10^{-10})
	x1 = time; x2 = [Cr]		X1 = time (h); X2 = [Cr] [μg L^{-1}]					
	x1	x2	X1	X2				
1	-1	-1	96	41.5	35.2	62.51	50.17	3.93
2	-1	1	96	48.5	28.4	38.41	69.39	12.99
3	1	-1	168	41.5	15.9	51.30	59.11	0.40
4	1	1	168	48.5	10.5	70.93	43.47	1.90
5	0	0	132	45.0	32.4	68.46	45.44	0.97
6	0	0	132	45.0	32.1	89.86	28.38	0.88
7	-1.41	0	81	45.0	29.0	56.49	54.97	7.22
8	0	-1.41	132	40.0	24.8	52.21	58.39	2.70
9	1.41	0	183	45.0	28.4	81.88	34.74	0.47
10	0	1.41	132	50.0	12.2	44.87	64.24	1.01

The response contour curves (Figure 1) were plotted for studying the effects of chromium concentration and exposure time in *P. subcapitata* in order for bioaccumulation to be evaluated. Each contour curve represents an infinite number of combinations of two test variables with the others when their respective zero level is maintained. We noticed that, when the metal is introduced to the culture medium, bioaccumulation is more intense in the first hours of exposure (81h) and for the intermediate chromium concentration (45 µg L^{-1}) (Figure 1). During the experiment, bioaccumulation shows a tendency to decrease.
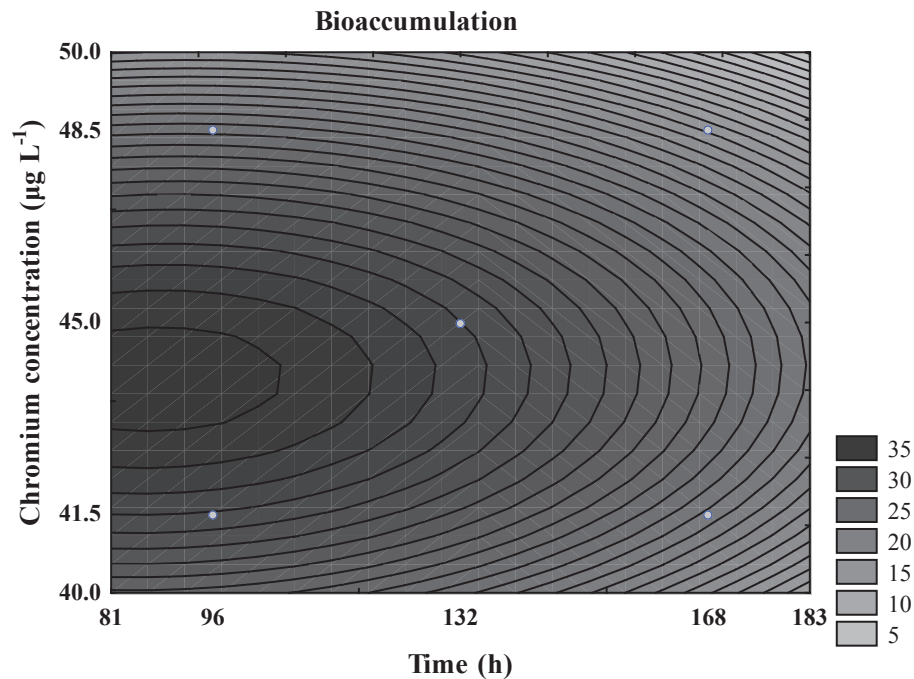
Figure 1. Contour plot of metal bioaccumulation in algae (µgCr gDW^{-1}) as a function of chromium concentration (40.0-50.0 µg L^{-1}) and exposure time (81-183 h) for *P. subcapitata*.

Bioaccumulation may decrease as a result of a diminution of permeability, active accumulation and absorption surfaces; while active excretion may play an important role (ALBERGONI et al., 1980). A number of physicochemical factors can influence the uptake of heavy metals by algae, such as light, pH, temperature, and chelating agents (PHILLIPS,

1995). The bioaccumulation level depends on the nature of the chemical compound, algae species, length of exposure, concentration in water (IVANCIUC et al., 2006). The bioaccumulation of chemical compounds in aquatic organisms represents important criteria for ecotoxicological evaluation and hazard assessment (MACKAY; FRASER, 2000; VOUTSAS et al., 2002).

Table 1 also shows the results obtained for biovolume, its relation to the bioaccumulated metal, and the experimental design that was used to investigate the influence of chromium on bioaccumulation and biovolume in *P. subcapitata*. Biovolume was affected and the reduction ranged between 28% and 69% in all treatments, in relations to control cells (data not showed). Biovolume was the highest at 45 µg L^{-1} Cr, however such values were recorded for higher exposure times at 132 and 183h (Figure 2).

Figure 2. Contour plot of algal biovolume as a function of chromium concentration (40.0-50.0 µg L^{-1}) and exposure time (81-183 h) for *P. subcapitata*.

The greater percentage of biovolume reduction (Figure 3) in relation to controls was obtained for more elevated concentration (48.5-50.0 µg L^{-1} Cr) and longer exposure time (168 h). A well documented case of morphological alterations has been reported for the predominant unicellular *Scenedesmus acutus* in the presence of chromium (VI) (CORRADI; GORBI, 1993). Peña-Castro et al. (2004) studying morphotypes in chromium stressed cultures of *Scenedesmus incrassatulus,* despite of not studied biovolume, were observed cell dimensions changes whose were significantly different from the control for length and width. Other side, Hawkins et al. (2005) tested the effect of Lugol's Iodine on the cell biovolumes of four common freshwater microalgae, and the maximum shrinkage in each species was a 30–40% reduction compared to the live cell biovolume.
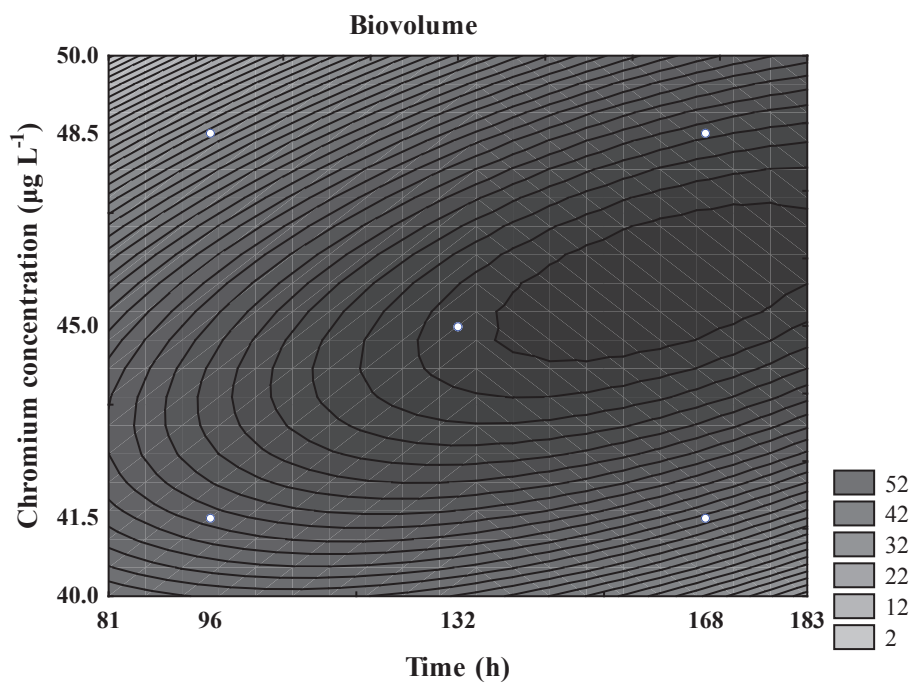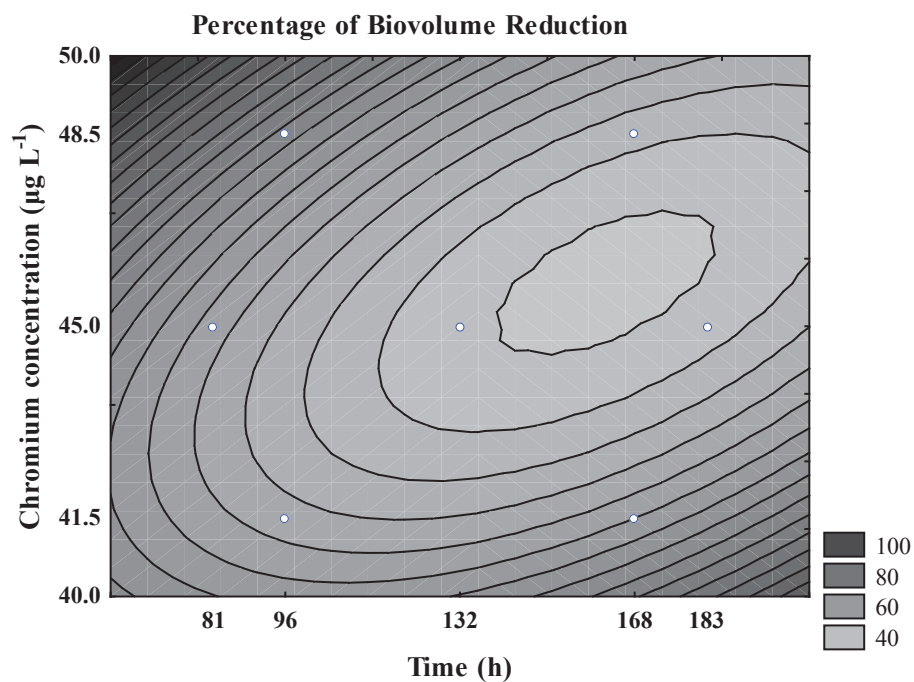

Figure 3. Contour plot of percentual biovolume reduction as a function of chromium concentration (40.0-50.0 µg L^{-1}) and exposure time (81-183 h) for *P. subcapitata*.

The average algal biovolume in the control measured in this study was 62.73 μm^3 (standard deviation ± 21.3), which was smaller than those examined by Weiner et al. (2004) to *P. subcapitata* at 74.49 μm^3. The highest percentual biovolume reductions in relation to the control were found in chromium concentrations above 48.5 μg L^{-1} Cr and shorter exposure time (Figure 3). Although the tested cells of *P. subcapitata* have experienced considerable reductions in biovolume in this study, Rodgher (2008, dates unpublished) reported increased biovolume for the more elevated concentrations of chromium in comparison to the control. Several authors using optical and/or electron microscopy (BOLAÑOS et al., 1992) and flow cytometry (FRANKLIN et al., 2001) have previously found an increase in cell volume of several species of microalgae in response to toxic levels of metals.

The enhanced bioaccumulation could be related to the observed reduction in biovolume. The highest ratios between the metal accumulated by algal cells and biovolume were found for the shorter exposure times and the elevated chromium concentrations (Figure 4).

The results for biovolume reduction obtained in this study may suggest that smaller cells represent a greater surface area and amplify metal binding sites. Dabbagh et al. (2007), working with ^{90}Sr bioaccumulation in filamentous cells of *Oscillatoria homogenea*, suggested that increased biomass caused an increment in binding sites and, therefore, in the bioaccumulation capacity. In addition, studies developed by Weiner et al. (2004) verified that both atrazine uptake and the cellular characteristics of microalgae (*Isochrysis galbana*, *Dunaliella tertiolecta*, *Phaeodactylum tricornutum*, *Pseudokirchneriella subcapitata*, and *Synechococcus* sp., listed in order of increasing sensitivity) indicated that smaller cells with greater surface area to volume ratios will incorporate more atrazine, and in general, will be more sensitive to atrazine exposure. According to Dönmez et al (1999), once the metal ion has diffused to the cell surface, it will bind to sites on the cell surface which exhibit some

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chemical affinity for the metal, and a number of passive accumulation processes may occur, including adsorption, ion exchange, coordination, complexation, chelation and microprecipitation.

Relation Chromium/Biovolume
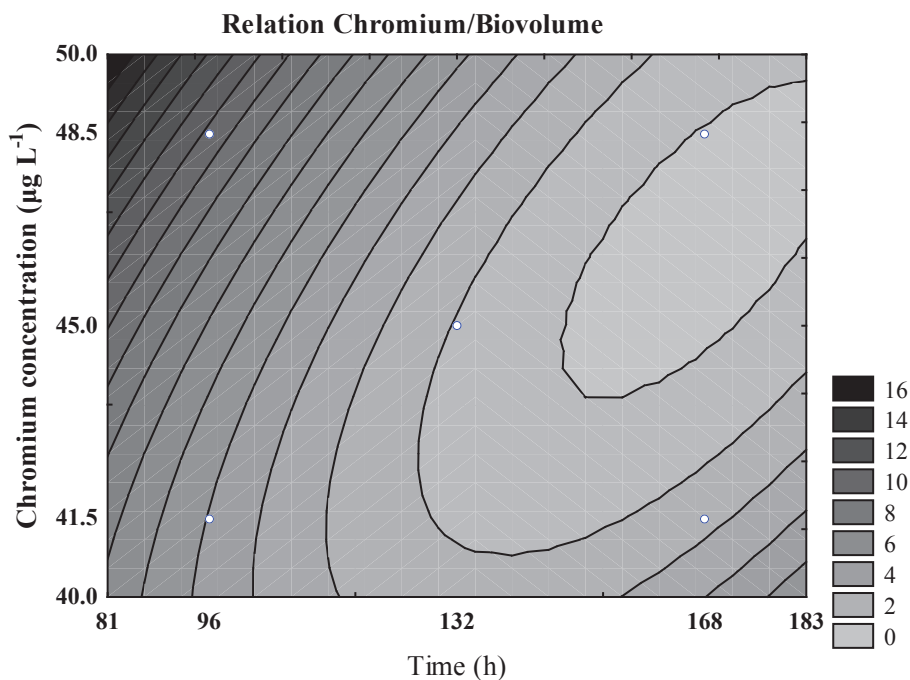
Figure 4. Contour plot of the ratio between metal accumulated by algal cells and biovolume, as a function of chromium concentration (40.0-50.0 µg L^{-1}) and exposure time (81-183 h) for *P.subcapitata*.

Studies by Rodgher & Espíndola (2008) found that *P. subcapitata* removed small amounts of chromium from the solution. Travieso et al. (1999) also observed small removal of chromium by the green algae *Senedesmus acutus* and *Chlorella vulgaris*, compared to other metals. In addition, Cervantes et al. (2001) suggested that both chromate and dichromate are negatively charged, and there is a limited chance of it being adsorbed by organic materials. A few studies describe Cr (VI)-reducing activities in fungi and plants (but not in algae yet) and the possible relationship of this process with chromate resistance and bioremediation.

On the other hand, Giloni-Lima et al. (2010) observed consistent algal growth inhibition was for the highest chromium concentrations (48 – 50 μgCr L^{-1}) and the longest exposure time (168 – 183h), where the bioaccumulation of chromium was greater. These results are consisted with findings by Labra et al. (2007) and Pereira et al. (2005), who identified consistent growth inhibition and reduction in the number of viable cells, which suggests that potassium dichromate is a strong algal cell pollutant and *P. subcapitata* is a sensitive organism suitable for monitoring the presence of chromium in water.

Model fitting and simulations

A model fitting was performed for the experimental design. The independent and dependent variables were fitted to the second-order model equation and examined in terms of goodness of fit. The ANOVA were used to evaluate the adequacy of the fitted model. The *R*-squared value provided a measurement of how much of the variability in the response values could be explained by the experimental factors and their interactions.

On the basis of ANOVA, a second order model (Equation 2) was established, describing the bioaccumulation, the biovolume and the ratio between the metal accumulated by algal cells and the biovolume, and the percentage of biovolume reduction, as a function of chromium concentration and exposure time. The model coefficients estimated by linear regression are shown in Table 2. The ANOVA for the regression model demonstrates that the model is highly significant especially for percentage of biovolume reduction, bioaccumulation and biovolume as it becomes evident from the Fisher's *F*-test (Table 2). The computed *F*-value for the models was higher than the tabular *F*-value (at the 5% level), indicating that the differences in treatment are highly significant.

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The elevated values of R^2, with variations between 0.77 and 0.88, demonstrate that 77 to 88% of the variability in the response could be explained by the model and suggests a satisfactory representation of the process model (HECK et al., 2005). In addition, there was a good correlation between the experimental and predicted values (data not shown).

The Student's t-test and p-values were used to check the significance of each coefficient (data not shown) which, in turn, is necessary to understand the patterns of the mutual interactions between the test variables (HECK et al., 2005). The terms of the second-order model, C (quadratic) for bioaccumulation, biovolume and percentage biovolume reduction were significant ($p<0.05$) and the interaction of exposure time and chromium concentration (t x C) for biovolume and percentual biovolume reduction was significant ($p<0.05$), which indicates that they act as limiting factors and that even small variations in their values will alter the bioaccumulation and the biovolume to a considerable extent. For the ratio between the metal accumulated by algal cells and the biovolume, only the t-linear term was significant with $R^2 = 0.78$, and the F ratio (F-value/F-value tabular) was 2.62.

Table 2. Obtained model and regression coefficients for Equation (1), and analysis of variance (ANOVA) for the experiments.

Term	Coefficient estimate (± Standard deviation)			
	Bioaccumulation (µgCr gDW⁻¹)	Biovolume (µm³)	Biovolume Reduction (%)	[Cr] per Biov (µg µm⁻³x10⁻¹⁰)
b_0	*32.055 (± 3.38)*	*79.160 (± 5.90)*	*36.905 (± 4.70)*	0.925 (± 1.99)
b_1	-4.746 (± 2.07)	7.152 (± 2.95)	-5.699 (± 2.35)	*-3.020 (± 0.99)*
b_2	-1.955 (± 2.46)	-5.755 (± 3.90)	4.587 (± 3.11)	1.948 (± 1.31)
b_{11}	-3.756 (± 2.07)	-1.857 (± 2.95)	1.481 (± 2.35)	1.020 (± 0.99)
b_{22}	*-7.050 (± 2.46)*	*-16.078 (± 3.90)*	*12.815 (± 3.11)*	0.953 (± 1.31)
b_{12}	0.332 (± 2.92)	10.933 (± 4.17)	-8.713 (± 3.32)	-1.887 (± 1.41)
P-value	0.0002	0.0001	0.0014	0.0387
R^2	77.0	88.3	88.3	78.0
F-value	33.10	26.40	60.34	12.44
F statistic table	5.12	4.74	5.32	4.74
F ratio[a]	5.88	5.57	11.34	2.62

The values in bold and italic are significant $p < 0.05$, with confidence level 95%. [a]F ratio (F-value/F-value tabular).

The generated models were used to run the simulations of bioaccumulation and biovolume (Figures 5a and b). The bioaccumulation was smaller in the more elevated time of exposition (168 and 183 h) and bigger in the smallest time (81, 96 and 132 h).
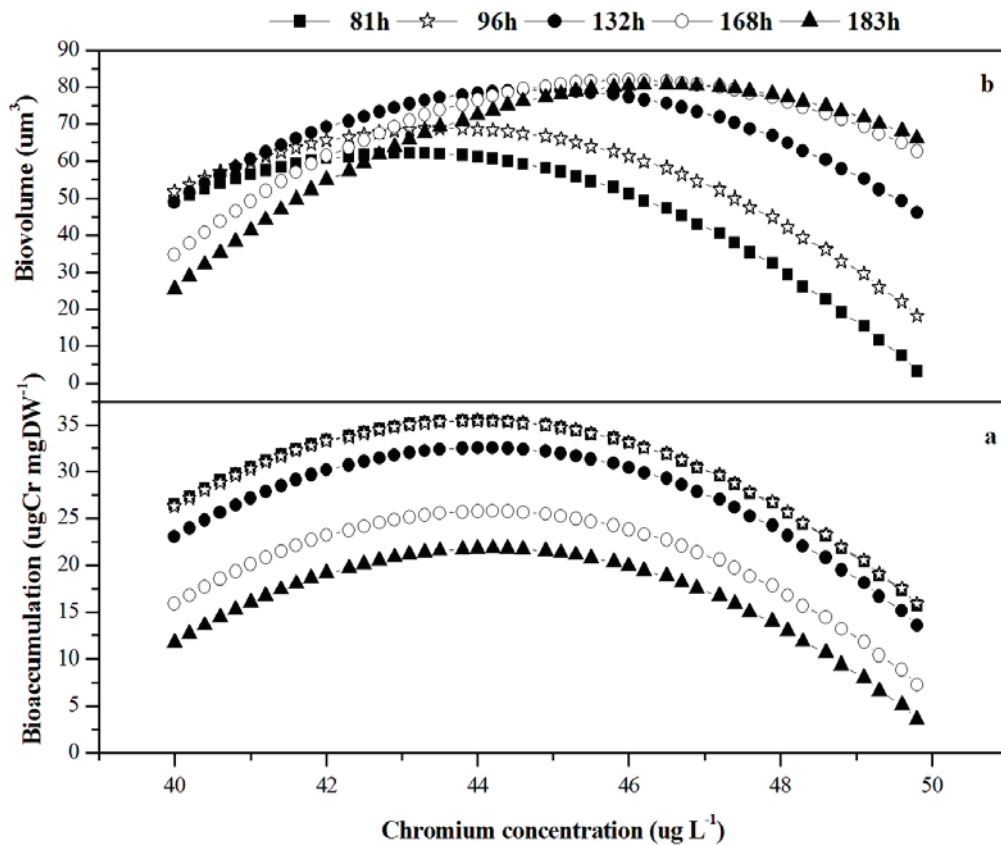
Figure 5. Simulations of (5a) bioaccumulation (μgCr gDW^{-1}) and (5b) biovolume (μm^3) based on the model parameters which were estimated from the experimental data.

Figure 5b shows that for the lower concentrations (40.0 and 41.5 μg L^{-1}), there was an increase in the biovolume until, approximately, 110h of exposure to the metal, after which time a decrease was observed. For the center point (45 μg L^{-1}) this behavior persisted until after approximately 155h of exposure. The smallest biovolume measurements were obtained for the shortest exposure times and for the highest chromium concentrations.

Although, De Schamphelaere et al. (2003) related that more research is needed do mechanistically understanding the relationships observed in the copper toxicity for *P. subcapitata*, the model developed by CCD has a high predictive capacity and will help improve the ecological relevance of current risk assessment. Park et al. (2009) studying combined effects between pH, DOC (Dissolved Organic Carbon) and hardness on acute metal toxicity, developed the empirical models able to predict in *D. magna* acute toxicity of natural waters and wastewaters containing Cu(II) or Cr(VI) as toxicants.

5.4. CONCLUSIONS

Chromium as dichromate is a pollutant that affects algal biovolume in *P. subcapitata*, and it is possible that this factor causes increased bioaccumulation of metal even in shorter exposure time. Our results suggest that smaller algal cells amplify the metal binding sites, increasing bioaccumulation and consequently their capacity to retain chromium. This hypothesis could justify the recommendation of *P. subcapitata* as a suitable organism for bioremediation. The CCD and the RSM are useful tools in order to asses how exposure time and chromium (VI) concentration affect bioaccumulation in *P. subcapitata*.

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CAPÍTULO 6. PAM FLUOROMETRY IN THE DETERMINATION OF THE SENSITIVITY OF *PISTIA STRATIOTES* TO CHROMIUM BY CENTRAL COMPOSITE DESIGN (CCD) AND RESPONSE SURFACE METODOLOGY (RSM)

ABSTRACT

The use of chlorophyll fluorescence through PAM fluorometric method permits greater ecological relevance of ecotoxicological tests. In this sense, the purpose of this study was to evaluate the chronic toxicity of chromium in *Pistia stratiotes* by Response Surface Methodology (RSM) and the PAM fluorometric method. Were also evaluated in the bioaccumulation of chromium, which was more intense in the root. The models in the analysis of pigment content revealed an increase in initial concentration, and subsequent reduction as it increased the concentration and time of exposure to chromium. Among the parameters of chlorophyll fluorescence analysis, the quantum yield of chlorophyll (Yield) and vitality index (R_{fd}) were more sensitive to chromium stress in *P. stratiotes*. We suggest intensified studies involving the use of PAM fluorometric method to expand the information on the influence of metals on chlorophyll fluorescence.

Keywords: bioaccumulation; experimental design; fluorescence chlorophyll; macrophytes; metal; water lettuce.

RESUMO

Método PAM Fluorométrico na determinação da sensibilidade de *Pistia stratiotes* ao Cromo pelo Delineamento Composto Central (DCC) e Metodologia de Superfície de Resposta (MSR)

O uso da fluorescência da clorofila através do método PAM fluorométrico permite ampliar a relevância ecológica dos ensaios ecotoxicológicos. Neste sentido o objetivo deste trabalho foi avaliar a toxicidade crônica do cromo em *Pistia stratiotes* através da Metodologia de Superfície de Resposta (MSR) e do método PAM fluorométrico. Também foram realizadas avaliações na bioacumulação do cromo, a qual foi mais intensa na raiz. Os modelos gerados nas análises do teor de pigmentos revelaram aumento nos tempos inicias, e posterior redução à medida que se elevaram as concentrações e o tempo de exposição ao metal. Dentre os parâmetros da fluorescência da clorofila analisados, o rendimento fotossintético da clorofila e o índice de vitalidade foram mais sensíveis ao estresse de cromo em *P. stratiotes*. Sugerimos que sejam intensificados os estudos envolvendo o uso do método PAM fluorométrico no sentido de ampliar as informações sobre a influência de metais na fluorescência da clorofila.

Palavras-chave: alface d´àgua, bioacumulação, desenho experimental, fluorescência da clorofila, macrófitas, metal.

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Chlorophyll fluorescence analysis has become one the most powerful and widely used techniques available to plant physiologists and ecophysiologists (MAXWELL; JOHNSON, 2000). That is because during the past decade, the measuring techniques for using variable chlorophyll fluorescence in non-invasive studies of photosynthesis have been refined significantly and measurements can now be undertaken at scales ranging from single chloroplasts and cells to microalgae, macroalgae and higher plants (GRUNVALD; KÜHL, 2004). During the last years a new type of pulse-amplitude modulated chlorophyll fluorometer (PAM) which enables high resolution fluorescence measurements (KÜSTER; ALTENBURGER, 2007) has been developed; the method has been used as a sensitive and rapid one for assessing toxic effect of pollutants in microalgae (JUNEAU et al., 2001, 2002, 2003) macrophytes (KÜSTER; ALTENBURGER, 2007; JUNEAU et al., 2003) and plants (JUNEAU et al., 2002). In order to gain useful information about the photosynthetic capacidade of a plant from measurements of chlorophyll fluorescence yield, it is necessary to be able to distinguish between the photochemical and non- photochemical contributions to quenching. Changes in the efficiency of photochemistry and the efficiency of heat dissipation (i.e. non-photochemical quenching) can occur depending on various internal and external factors (MAXWELL; JOHNSON, 2000). In this context, it has been shown itself very interesting in the practical application of chlorophyll a (Chl *a*) fluorescence as a rapid and sensitive bioindicator of plant stress in response to different chemical factors (KÜSTER; ALTENBURGER, 2007; JUNEAU et al., 2003; MALLAKIN et al., 2002; KRAUSE;WEIS 1984).

Other aspect that has been approached during the last years and that has been often discussed about was the miniaturization of toxicity test systems for rapid and parallel measurements of high amounts of samples (KÜSTER; ALTENBURGER, 2007). Due to he fact that tests systems for aquatic macrophytes are still consuming much time (due to low growth rates of thalli), and to the large water volume and high amounts of toxicants that have to be added to achieve effective test concentrations on aquatic macrophyte test systems, fast analysis of toxicants on aquatic macrophytes has not been established yet and little data is available in this type of assessing.

The ecosystems are constituted of biotic and abiotic factors which interact between them. The introduction of inorganic poluttants in these ecosystems, such as metals for example, amplifies even more the existent interactions. Towards a new way of choosing experiments efficiently and systematically, in order to give reliable and coherent information, emerges the statistical design through the response surface methodology (RSM) and the use of experimental design of the Central Composite Design (CCD) (FURLANETTO et al., 2003). To develop studies of ecotoxicologic in aquatic ecosystems, this methodology represents a powerful technique for the investigation of multivariate systems, once it makes possible to analyze the direct effects of test variables that interfere in this processes and their interactions using a minimum number of combinations, besides reducing the number of experiments and repetitions without losing statistic confidence (since it was possible to calculate the experimental error).

The objective of this work was to propose the use of response surface methodology in assessing chronic toxicity of chromium (Cr) for *Pistia stratiotes* through chlorophyll *a* fluorescence. This study also investigated the combination of RSM and PAM-fluorometry as

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indicators of rapid and sensitive environmental stress assessment and their reliability on aquatic macrophyte test systems.

6.2. MATERIAL AND METHODS

Macrophytes culture

The macrophytes *Pistia stratiotes* L. was obtained from São Paulo littoral and were maintained at the Laboratory of Ecotoxicology and Ecophysiology of Aquatic Organisms of the Water Resources and Applied Ecology Center (CRHEA) of the University of São Paulo (São Paulo State, Brazil). The macrophytes were cultivated in containers (1000 L) with tap water and the NPK (4:14:8) fertilizer. The solution of cultive was maintained at pH 7 by titration with dilute HCl or NaOH.

Toxicity tests

Plants with similar size and growth stage were selected, washed with tap water and acclimated for two days in laboratory in nutrient solution containing (mM): 1.25 $Ca(NO_3)_2.4H_2O$, 1.5 KNO_3, 0.5, KH_2PO_4, 0.5 $MgSO_4.7H_2O$, 0.05 H_3BO_3, 0.01 $MnCl_2.4H_2O$, 0.30 $CuSO_4.5H_2O$, 0.20 Na_2MoO_{24} and 0.5 NaFeEDTA(10%Fe) (ODJEGBA; FASIDI, 2004).

Tests were performed with three plant in 1 liter nutrient solution submitted to different concentration of chromium (1.0-6.0 mg Cr L^{-1}) and that was supplied as $K_2Cr_2O_7$, and which exposure time was 12-60 h (Table 1).

Table 1. Process variables used in the CCD showing the treatment combinations between chromium concentration and exposure time in *P. stratiotes*.

Runs	Coded setting levels		Actual levels	
	x1= time; x2= [Cr]		X1= time (h); X2= [Cr] (mg l^{-1})	
	x1	x2	X1	X2
1	-1	-1	19	1.7
2	-1	1	19	5.3
3	1	- 1	53	1.7
4	1	1	53	5.3
5	0	0	36	3.5
6	0	0	36	3.5
7	0	0	36	3.5
8	0	- 1.41	36	1.0
9	0	1.41	36	6.0
10	-1.41	0	12	3.5
11	1.41	0	60	3.5

Plants in nutrient solution without chromium were the control. Both the control and the treated solutions were maintained at pH 5.5 by titration with dilute HCl or NaOH; such low pH helps to keep metal in solution and available for absorption by plant roots (ODJEGBA; FASIDI, 2004). The macrophytes during the test were maintained at a 26±2 °C and under a constant irradiance of 7,000 lux provided by cool-white fluorescent lamp.

Fluorescence measurements

Chlorophyll fluorescence measurements were performed in PAM fluorometer (Mini PAM Walz 2007F-1, Germany) that were placed directly in macrophytes leaves clip. The

constant fluorescence (F_0) of a dark-adapted plant was measured by using modulated light having a very low intensity to avoid reduction of the PSII primary electron acceptor Q_A. Prior to the fluorescence measurement, leaves were adapted to darkness during 30 minutes in order to induce an equilibrium state of the photosynthetic electron transport. Maximal fluorescence yield (F_m) was induced by a short saturating pulse of light which triggers the reduction of all Q_A. The change of the fluorescence yield (F) during the following illumination by actinic light induced the typical Kautsky effect. Simultaneously, the change of the maximal fluorescence yield (F'_m) was induced by saturating pulses given periodically (JUNEAU et al., 2003). From each of these, a value for F'_m the fluorescence maximum in the light, could be measured (MAXWELL; JOHNSON, 2000). The actinic light and saturating light pulse intensities were 160 μmol m^2/s and 2000 μmol m^2/s, respectively. At the steady state of electron transport, actinic light was turned off and a far-red light was applied to ensure rapid and complete oxidation of Q_A (JUNEAU et al., 2003). The steady state value of fluorescence immediately prior the flash was termed F_t. Photochemical quenching parameters always relate to the relative value of F'_m and F_t. The most useful parameter was the one that measured the efficiency of Photosystem II photochemistry, Φ_{PSII} (GENTY et al., 1989). This is calculated as:

$$\Phi_{PSII} = (F'_m - F_t)/ F'_m \tag{1}$$

Another utilized fluorescence parameter was the ratio decrease fluorescence (R_{df}) that allows interpretation to the photosynthetic process; it is also designed to vitality index and can be used to evaluate the cycle Calvin activity and its related process (LICHTENTHALER et al., 1986; LICHTENTHALER; RINDERLE 1988). This parameter is calculated as:

$$R_{df} = (F_m - F_t)/F_t \tag{2}$$

The values of R_{df} superior to 2.5 indicated a good functionality of photosynthetic activity and the values above 1.0 suggested that the CO_2 fixation could be severity compromised (LICHTENTHALER et al., 1986; LICHTENTHALER; RINDERLE 1988).

Chlorophyll Analysis

Leaves discs of *P. stratiotes* were cut with ca 200 mm^2 surface. These discs were extracted with 2 mL aqueous acetone 80% in a mortar with pestle. The homogenate, combined with a further three washings of the pestle and mortar (each of 1.5 mL) with the same solvent, was centrifuged at 1500 rpm for 10 min. The pellet was then extracted with a further 1 mL of solvent and the pooled supernatants adjusted to a final volume of 8 mL. The absorbance at the major absorption peak of chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) and Chlorophyll total (Chl *a* + Chl *b*) were measured in Spectrophotometer and the concentrations in µg L^{-1} were then calculated using the equations (3, 4 and 5) described below (PORRA et al., 1989):

$$\text{Chl } a = 12.25 \text{ A}^{663.6} - 2.55 \text{ A}^{646.6} \tag{3}$$

$$\text{Chl } b = 20.31 \text{ A}^{646.6} - 4.91 \text{ A}^{663.6} \tag{4}$$

$$\text{Chl } a + b = 17.76 \text{ A}^{646.6} + 7.34 \text{ A}^{663.6} \tag{5}$$

Metal analysis

At the end of the each exposure time, leaves and root samples of *P. stratiotes* were taken to determine the metal accumulated by plants. The samples were dried at 48 h to 60 ºC and submitted to acid digestion (HNO_3 and H_2O_2); all analytical procedure was accompanied

by analytical blank (APHA, 1995). All the samples were analyzed in triplicates by graphite-furnace atomic absorption spectrometry (Varian AA 220).

Experimental Design

Toxicity tests with *P. stratiotes* were conduced in 11 experiments for the study of two parameters (see matrix in table 1). The model studied is a 2^2 experimental design. It has been selected time of exposure (X1) and chromium concentration (X2). The experimental results of the response surface methodology were fitted with a second-order polynomial equation (6) by a multiple regression technique

$$Y = b_0 + b_1 \, x_1 + b_2 \, x_2 + b_{11} \, x_1{}^2 + b_{22} \, x_2{}^2 + b_{12} \, x_1 \, x_2 + \varepsilon \quad (6)$$

Y is the predicted response, b_0, b_1, b_2, b_{11}, b_{22}, b_{12} are constant coefficients, and x_1, x_2 are the coded independent variables or factors.

The test factors were coded according to the following regression equation (7):

$$x_i = \left(\frac{X_i - X_0}{\Delta X_i} \right) \quad (7)$$

where x_i is the *coded* value and X_i is the *actual* value of the ith independent variable, X_0 is the actual value at the center point, and ΔX_i is the step change value. Statistic 7.0 software (Statsoft, USA) was used for regression and graphical analysis of the data.

The quality of fitting of the second-order model equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by an *F*-test. The significance of the regression coefficients was tested by a *t*-test.

Chromium and pigment photosynthetic content

The results obtained from 2^2 factorial designs experimental to metal concentration and chlorophyll content in *P. stratiotes* are shown in Table 2. The plants exposed to different Cr concentrations accumulate the metal in their root system maintaining high level in root tissues in relation to leaves. The response analysis surface of the chromium concentration in roots and leaves was performed and the coefficient of determination was 75.0% in roots and 77.1% and leaves, verified the adequacy of using the regression model for describing the contour surface with 95% of confidence (Table 3 and Figures 1 and 2).

Table 2. The mean experimental design responses with the results obtained the chlorophyll content in $\mu g\ L^{-1}$ g f.wt.$^{-1}$, the fluorescence parameters (F_v, F_m, F'_m, Φ_{PSII}, R_{df} and NPQ), the chromium concentration in μg g dry wt.$^{-1}$ in roots and leaves.

| Run	Chl a	Chl b	Chl t	F_v	F_m	F'_m	Φ_{PSII}	R_{fd}	NPQ	[Cr] in roots	[Cr] in leaves
							Experimental design responses				
1	126.340	211.829	338.169	1826.22	2098.67	1228.00	0.634	5.49	0.71	81.200	1.074
2	114.516	217.225	331.741	579.98	2479.50	364.00	0.151	1.89	5.81	86.337	2.484
3	93.819	178.690	272.509	1192.18	1102.33	1065.00	0.278	0.90	0.04	87.453	0.841
4	129.254	244.269	373.523	1582.18	1780.33	1159.00	0.417	1.09	0.54	114.035	1.646
5	114.045	178.242	287.559	2005.14	2413.00	1057.50	0.367	2.11	1.28	92.148	9.317
6	114.400	177.975	288.340	1967.01	2424.25	1055.00	0.376	2.38	1.30	91.294	9.109
7	114.982	189.469	301.772	1986.07	2435.50	1056.25	0.372	2.25	1.31	90.440	9.524
8	118.940	201.104	320.044	377.28	2490.67	988.00	0.423	5.66	1.52	60.357	3.031
9	135.038	248.473	386.237	1663.36	2302.00	808.00	0.630	4.55	1.85	147.081	8.465
10	115.680	218.377	334.058	611.55	694.00	1026.00	0.319	2.27	0.00	56.439	3.386
11	112.591	217.879	330.470	1927.89	2355.50	389.00	0.461	2.70	5.06	137.312	4.973
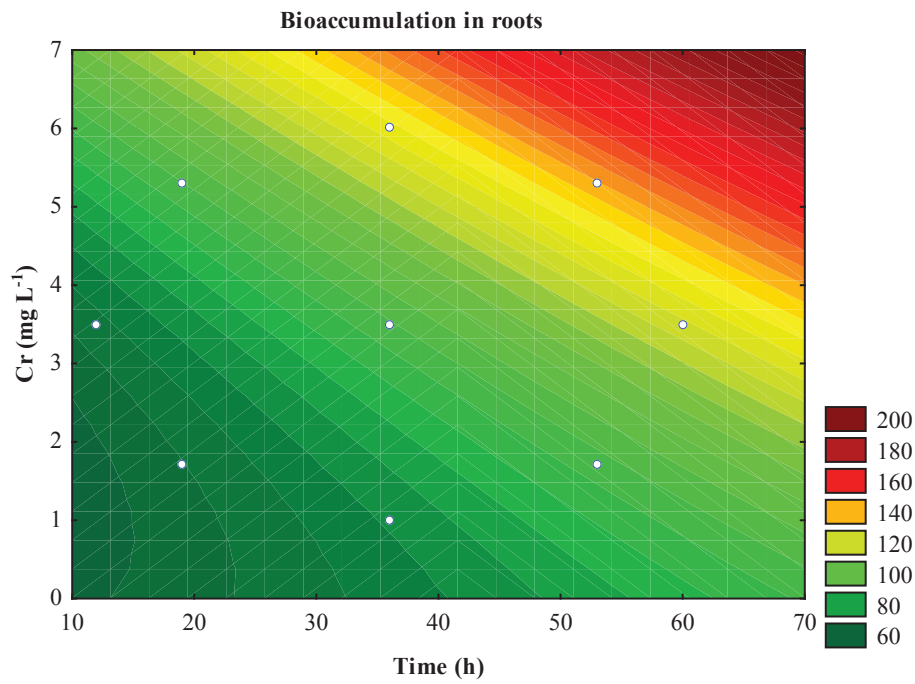
Figure 1. Contour surface plot of bioaccumulation in roots (µg gMS^{-1}) as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

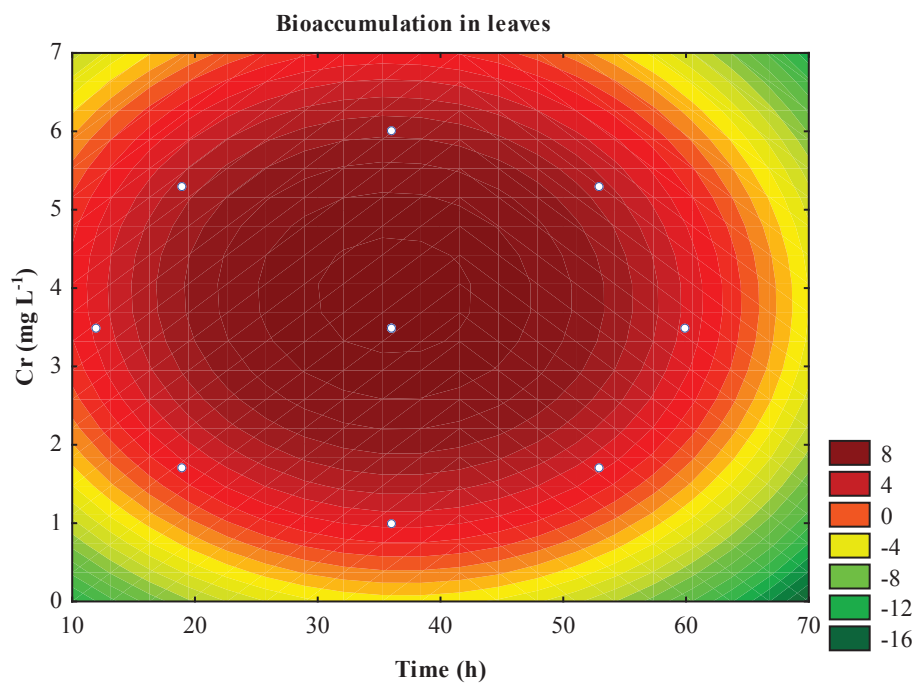
Figure 2. Contour surface plot of bioaccumulation in leaves (µg gMS^{-1}) as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

The time and chromium concentration linear were significantly different in roots, but in leaves those terms were significant in the quadratic model which represents that leaves were more sensible to metal concentration and that even small variations in their values will alter the bioaccumulation to a considerable extent. The increase of metal accumulation in roots occurred in more elevated chromium concentrations and after lengthier exposition (Figure 1).

On the other hand, the presences of curvature in the regions of the center point in the contour surface shows that the response to accumulation of metal in leaves was different (Figure 2). Uptake and accumulation of chromium by various plant species are well documented (SHANKER et al., 2005; VERNAY et al., 2007; GANESH et al., 2008). Paiva et al (2009) working with *Eichornia crassipes* and Sinha et al. (2009) with *Pistia stratiotes* revealed a higher concentration of chromium in the roots than in the shoots. Different mechanisms are proposed to explain the metal concentration gradient between the roots and shoots in the plants exposed to chromium mainly in hyperaccumulator plants. Soltan and Rashed (2003) reported that this accumulation could have happened due to the co-precipitation of metals in the plaques of iron (Fe) and manganese (Mn) on the roots. Otherwise, MacFarlane and Burchett (2000) related that most plants restricted metal transport across the root endodermis, and removed any mobile ions in the xylem by means of storage in cell walls and vacuoles, or binding by the metal binding proteins such as metallothionines or phytochelatins.

Table 3. Staristical analysis of different parameters analyzed by experimental design. The chlorophyll content, the fluorescence parameters (F_v, F_m, F'_m, Φ_{PSII}, R_{df} and NPQ), and the chormium concentration in roots and leaves.

Terms	Coefficient estimate ($\pm$ Standard deviation)										
	Chl a	Chl b	Chl t	F_v	F_m	F'_m	Φ_{PSII}	R_{fd}	NPQ	[Cr] in roots	[Cr] in leaves
b_0	*114.494 (2.1)*	*181.929 (4.0)*	*292.613 (6.1)*	*1315.449 (698.4)*	211.430 (715.6)	*1529.604 (453.8)*	*1.362 (0.2)*	*13.718 (3.1)*	-1.556 (2.2)	*9.330 (1.4)*	*91.326 (11.5)*
b_1	-2.776 (1.3)	-0.852 (2.5)	-3.628 (3.8)	*70.133 (25.7)*	*134.962 (26.4)*	-26.266 (16.7)	-0.014 (0.0)	*-0.305 (0.1)*	0.162 (0.1)	0.146 (0.8)	*18,553 (7.1)*
b_2	-1.341 (1.5)	*16.022 (2.9)*	*16.419 (4.5)*	*-1.525 (0.3)*	*-2.453 (0.3)*	0.054 (0.2)	-0.00008 (0.0)	0.002 (0.0)	-0.001 (0.0)	*-3.453 (1.0)*	0.755 (8.4)
b_{11}	*5.806 (1.3)*	*17.272 (2.5)*	*23.560 (3.8)*	153.432 (238.0)	-393.662 (243.9)	42.109 (154.6)	*-0.365 (0.0)*	-2.215 (1.1)	-0.134 (0.7)	1.238 (0.8)	*19.307 (7.1)*
b_{22}	*5.124 (1.5)*	*19.372 (2.9)*	*26.920 (4.5)*	-22.960 (28.3)	-17.450 (29.0)	*-59.922 (18.4)*	0.021 (0.1)	0.097 (0.1)	*0,314 (0.1)*	*-2.664 (1.0)*	4.198 (8.4)
b_{12}	*11.815 (1.8)*	*15.046 (3.5)*	*26.861 (5.3)*	2.428 (3.5)	*13.368 (3.6)*	*7.827 (2.3)*	*0.005 (0.0)*	0.031 (0.0)	*-0.038 (0.0)*	-0.151 (1.2)	5.361 (9.9)
R^2	0.943	0.962	0.955	0.940	0.940	0.888	0.909	0.835	0.940	0.771	0.749
F-value	38.8	37.6	31.8	63.1	141.5	31.6	40.0	45.7	141.8	13.5	11.9
F stat table	4.35	4.53	4.53	5.32	5.12	4.46	4.46	5.12	5.12	4.46	4.46
F ratio[a]	8.9	8.3	7.0	11.9	27.6	7.1	9.0	8.9	27.7	3.0	2.7

The values in bold and italic are significant $P < 0.05$, with confidence level 95%. [a]F ratio (F-value/F-statistic table).

Independent to mechanism used for to hyperaccumulate metals the consensus is that *Pistia stratiotes* is used for phytoremediation of wastewater or natural water bodies polluted with heavy metals, and that this specie exhibits different patterns of response to Ag, Cd, Cr, Cu, Hg, Ni, Pb and Zn, with almost all the elements being accumulated at high concentrations in the root system (SHAH; NONGKYNRIH, 2007).

The photosynthetic pigments in the plants are considered as one of the sensitive parameter under stress condition particularly metal toxicity (SINHA et al., 2005). Considering that, both increase and decrease in the chlorophyll content has been reported in different plant species exposed to Cr (VI) (SHARMA; SHARMA, 1993; SAMANTARAY et al., 2001). The chlorophyll content (Figures 3-5) was affected by more elevated chromium concentrations situation where the values were smaller as the control plants, mainly the Chl *a*.
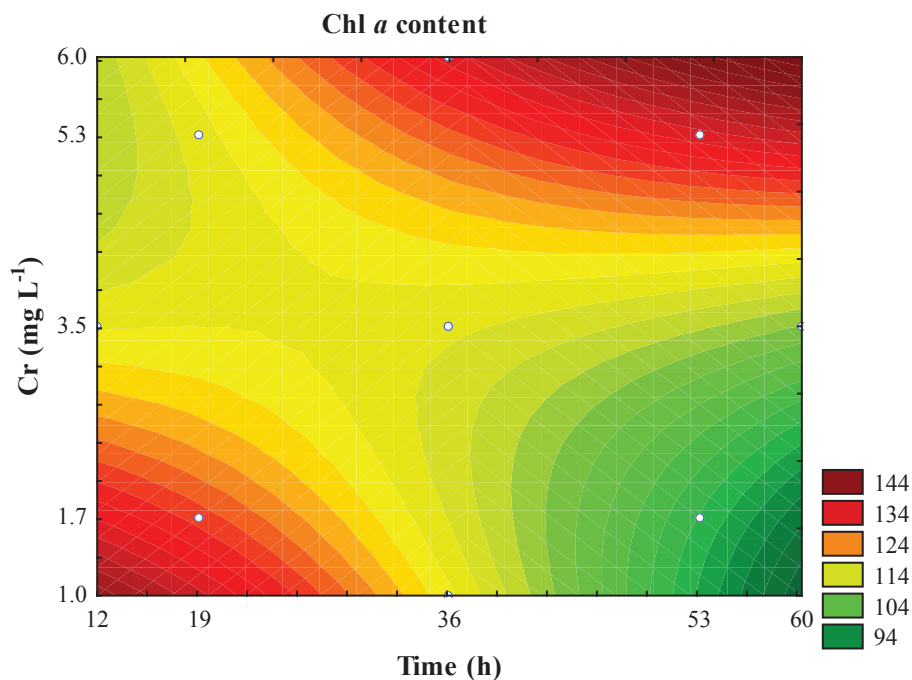

Figure 3. Contour surface plot of chlorophyll *a* content as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

The linear and quadratic terms to chromium concentration and their interaction with exposure time were significantly different to chlorophyll content ($p<0.05$). The exposure time quadratic term was also significantly different to Chl *b* and total (Table 3, Figures 3-5). The significant quadratic terms represent that they act as limiting factors and even small variations in their values alter the chlorophyll content to a considerable extent. The response surface analysis of chlorophyll content was performed and the elevated coefficient of determination 94.33; 96.16 and 95.5% to Chl *a, b* and total, respectively, revealed the adequacy of using the regression model to describe the contour surface (Figure 3-5). In the contour surface (Figure 3) it is possible to observe a decrease to Chl *a* content in the more elevated chromium concentrations and more prolonged exposure time (presence of curvature in the region of interest).
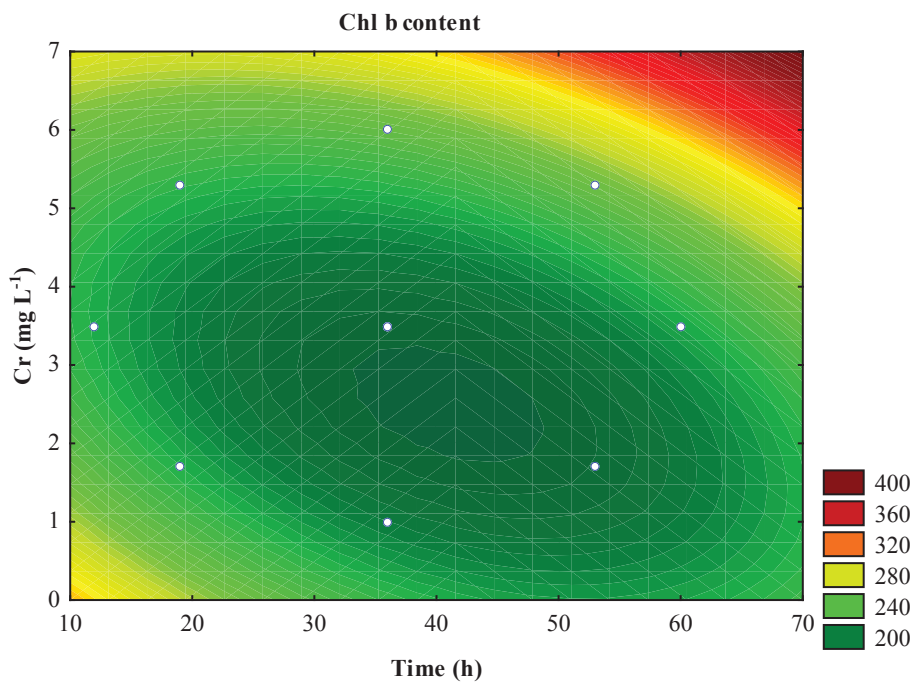

Figure 4. Contour surface plot of chlorophyll *b* content as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.
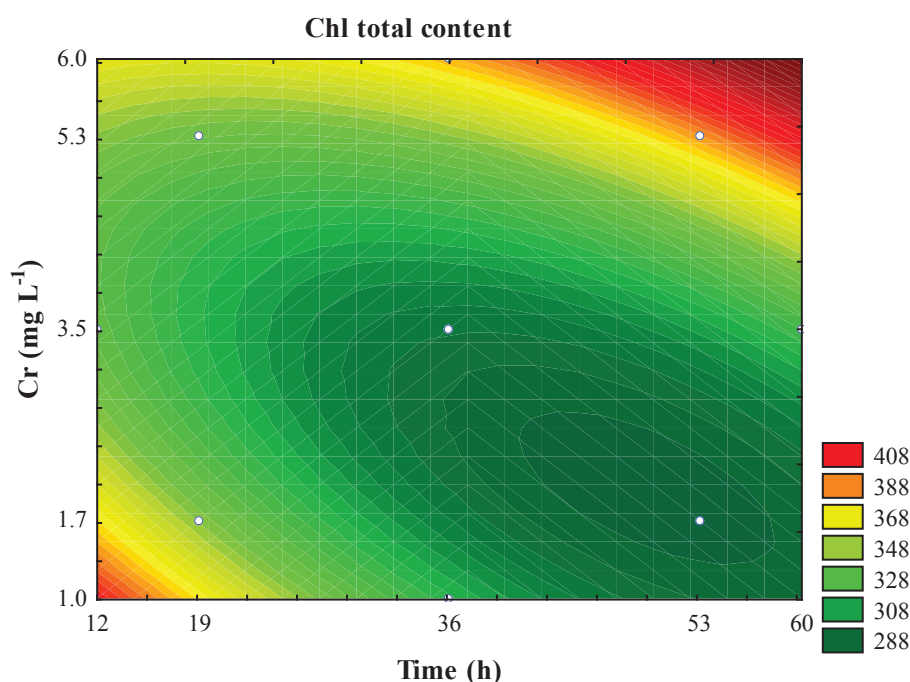
Figure 5. Contour surface plot of chlorophyll total content as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

Boonyapookana et al. (2002) related that a decrease in photosynthesis in plants, promoted by increased Cr concentrations in nutrient solution, is associated with biochemical changes, causing inhibition of chlorophyll synthesis. Decreases in total chlorophyll have been well documented under Cr stress, and in general the chlorophyll *a* was more sensitive than chlorophyll *b* (VAJPAYEE et al., 2000; PANDA; CHOUDHURY, 2005; SINHA et al., 2005; PAIVA et al., 2009). Probably, as *Pistia stratiotes* is a hyperacumulated and exhibits high tolerance index a various metals (SHAH; NONGKYNRIH, 2007), it can support high concentrations of metals by longer expositions before being affected by the chlorophyll content.

Chlorophyll a fluorescence parameter analysis

The measurements of chlorophyll *a* fluorescence parameters were analyzed from 2^2 factorial designs experimental in *P. stratiotes* submitted to different chromium concentrations (Table 2). The maximal fluorescence all dark-adapted (F_m), the variable florescence (F_v), the maximal fluorescence light adapted (F'_m), the quantum yield of PSII ($\Phi PSII$) also designated Yield, the non-photochemical quenching (NPQ) and the vitality index (R_{df}) having good adjust in the regression of the model. The ratio F_v/F_m was significant only for the model based on pure error ($p < 0.05$). However, the initial fluorescence (F_o) has not good adjust and its result was not significant.

F_v is one of the parameters of the fast fluorescence kinetics and represents an increase of fluorescence from F_o until F_m. F_v values were lower at higher concentrations of chromium and longer time exposure; these results can be seen in the lighter regions of the graph of response surface (Figure 6). The values obtained to F_v showed that the linear and quadratic model of time and the interaction between time and chromium concentration were significantly different and the coefficient of determination to F_v was 94% ($p < 0.05$), verified the adequacy of model for describing the contour surface (Table 3). With increasing chemical concentrations, the variable fluorescence showed a decrease in relation to control plants that was accompanied by the decrease of the effective quantum yield. Similarly to what was observed by Paiva et al. (2009) the variable fluorescence was substantially diminished compared to control plants.

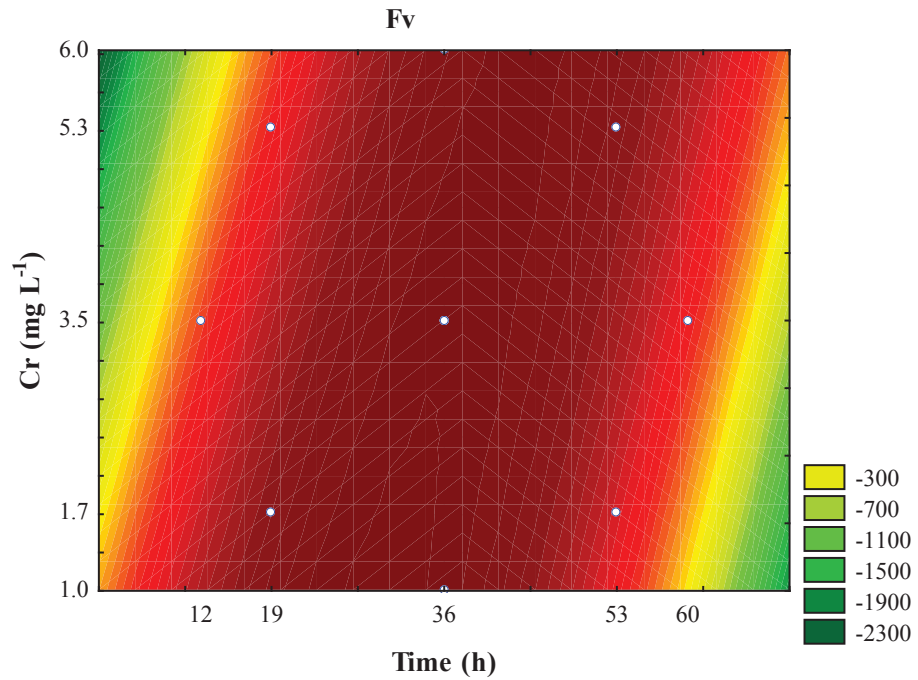Figure 6. Contour surface plot of variable fluorescence (F_v) as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

The proportion of radiant energy emitted in the form of fluorescence is low under plant optimum conditions. However, in many situations, fluorescence increases under stress conditions, and there are also changes in the characteristics related to fluorescence (PAIVA et al., 2009). On this premise, it was observed that the values were more elevated than in control plants in all the concentrations and exposure time of the study. Similarly to Fv, the values obtained to F_m showed that the linear and quadratic model of time and the interaction between time and chromium concentration were significant to 95%. A response surface analysis of maximal fluorescence presented the elevated coefficient of determination of 94% ($p<0.05$), verified the adequacy of model of regression (Table 3 and Figure 7). The reduction of F_v was observed after 53h of exposition to metal, when the values were smaller in control plants. These results can be observed by the presence of curvature in the regions of interest.

Figure 7. Contour surface plot of maximal fluorescence (F_m) as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

After the emission of pulses of actinic light, fluorescence kinetics becomes more complex. With the light pulses, it was possible to get varying levels of F_m, which is now called F'_m, and represents the dissipation of energy or quenching. Elevated values were observed during almost all the experiment. The quadratic factor of regression model to chromium and interaction between exposure time and chromium concentration were significant ($p<0.05$), what indicates more sensibility to these factors. The response surface of the F'_m was performed (Figure 8) and the coefficient of determination was 89%.
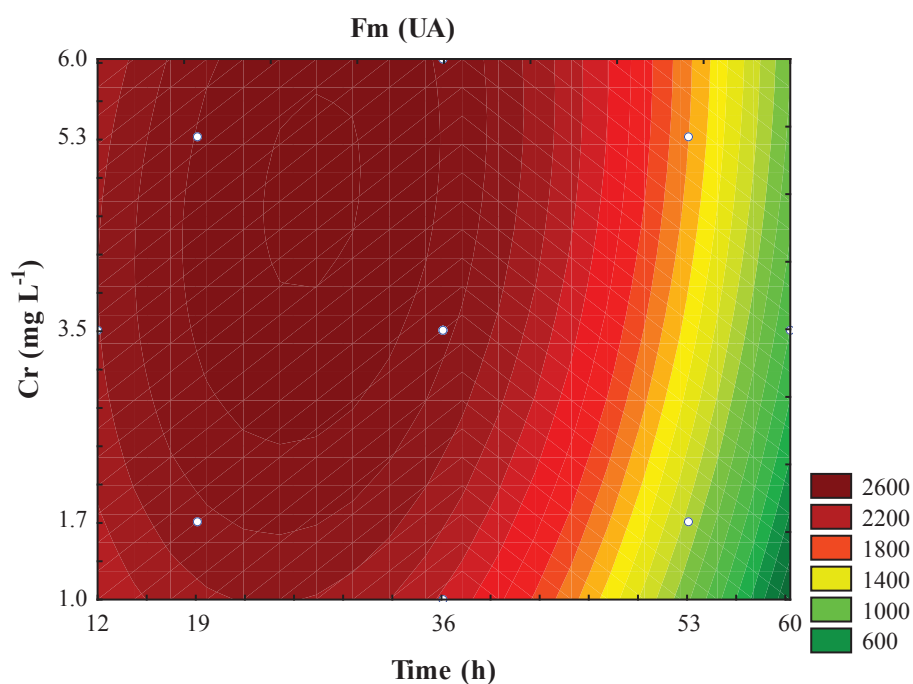
Figure 8. Contour surface plot of fluorescence (F'_m) as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

The difference between the maximal fluorescence all dark-adapted (F_m) and light adapted (F'_m) is denominated non-photochemical quenching (NPQ), what is based on the matrix model of organization of the antenna system. This parameter represents all forms of dissipations of energy, mainly thermal dissipation (BUTLER, 1980). More elevated NPQ values were observed in the bigger chromium concentrations already in the initial times of exposition, according to darker regions in the contour surface (Figure 9). The statistical analysis was performed and chromium quadratic model was significant to 95%, such as the interaction between chromium and exposure time, similarly to significant factors in F'_m. The coefficient of determination was 94%, indicating adequacy of using the regression of model for describing the contour surface (Table 3).
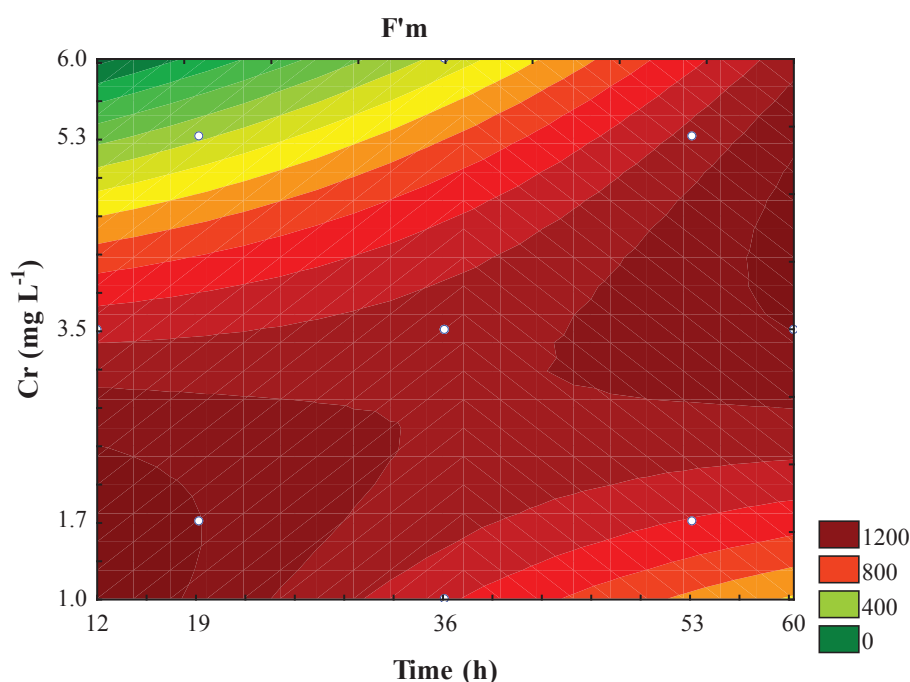
Figure 9. Contour surface plot of NPQ as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

Values Fv/Fm were between 0.72 and 0.89 (not showed data) what shows that, according to Jakl and Bolhàr-Nordenkampf (1991), values obtained were of Fv/Fm between 0.75 and 0.85. The model based pure error showed coefficient of determination of 81% where only the chromium quadratic model was not significant. This parameter presented values 1.0 in the 2, 3 and 8 treatments, which except to run 8, was coincident to values low photosynthetic yield.

The efficiency of Photosystem II photochemistry (ΦPSII) or Yield is measured during a saturation pulse which leads to full inhibition of energy conversion at PSII reaction centers and hence, transiently induces maximal fluorescence yield. Elevated values of yield were observed only in 1 and 9 runs, what corresponds to the 19h and 36h and 3.2 and 2.0 mgL^{-1} of chromium, respectively. Those results can be observed in the presence of curvature

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in the regions of interest in the response surface in the statistical analysis that was performed in figure 10.
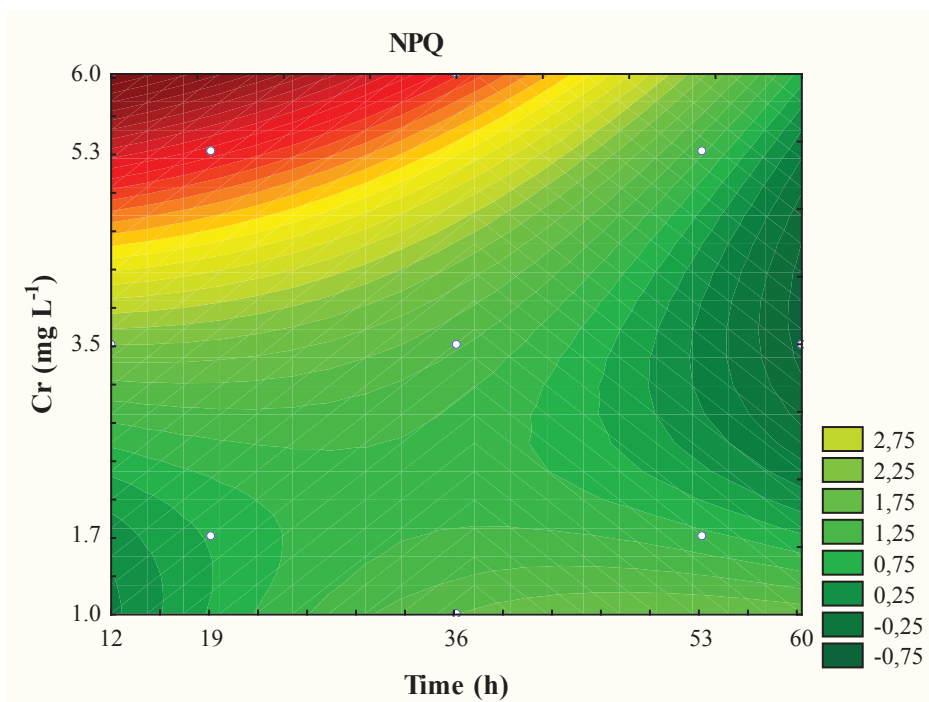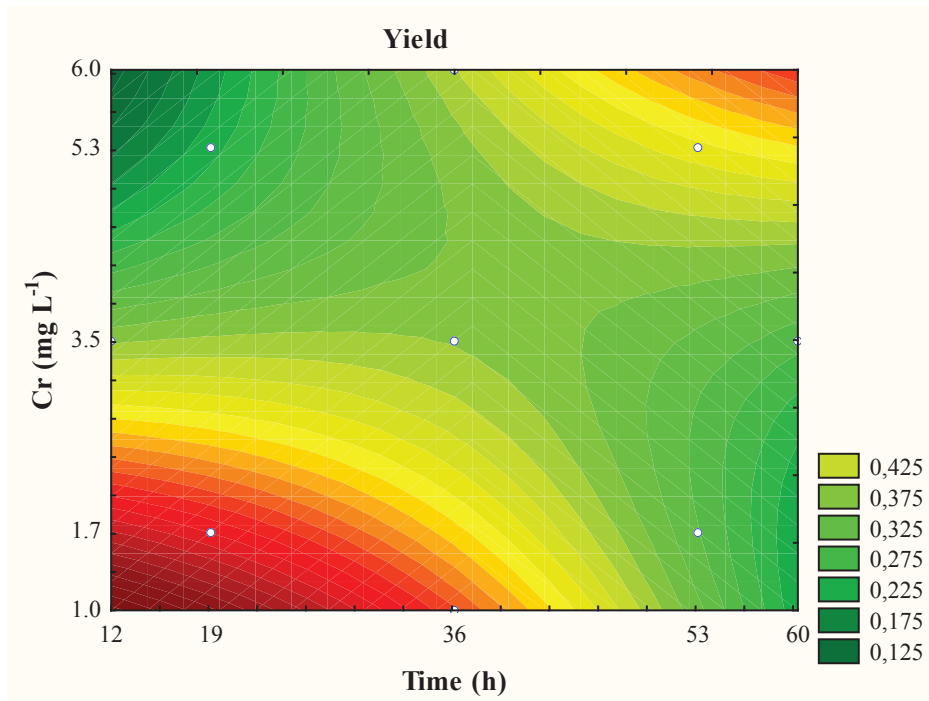

Figure 10. Contour surface plot of Yield as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

A response surface analysis of Yield reveals an elevated coefficient of regression (91%), verified the adequacy of using the regression of model for describing the response surface ($p<0.05$). In this model the chromium linear and the interaction between exposure time and chromium was significant (Table 2). Shanker et al. (2005) related that chromium stress can also affect photosynthesis in terms of CO_2 fixation, electron transport, photophosphorylation and enzyme activities. It is well accepted that yield of dissipated fluorescence will be significantly changed when photosynthetic or related biochemical or physiological processes are affected. This condition has direct relation with the kinetics of fluorescence emitted by higher plants or algae (KRAUSE; WEIS, 1984). Therefore, if

photosynthesis or dependent biochemical or physiological processes are inhibited, the yield and the kinetics of dissipated fluorescence will be significantly changed (JUNEAU et al., 2003) and the interference in the physiological process chromium-induced could explain the results observed in the reduction of maximal fluorescence yield.

Another indicator of the potential photosynthetic activity of a leaf is the ratio of fluorescence decrease (fd) to the steady state fluorescence fs, denominate vital index, where Rfd = fd/fs (LICHTENTHALER et al., 1986). The more elevated ratio decrease fluorescence values were observed in the reduced or intermediated chromium concentration as the exposure time also reduced (Table 2, Figure 11). The results observed in treatments 1 (5.49), 8 (5.66) and 9 (4.55), represents the plants with good functionality of their photosynthetic activity, according to Lichtenthaler and Rinderle (1988). In other treatments the results were included among the results $1.0 \leq R_{fd} \leq 2.5$. Values smaller than 1.0 represent that the CO_2 fixation is severely compromised, what occurred with treatment 3 and 4 (both with 53 h of exposure, 1.7 and 5.3 mg Cr L^{-1}, respectively). The statistical analysis was performed indicating that about 84% of the variability on the data could be explained by the regression model where only linear time was significant.

In terrestrial plants, the negative action of Cr on photosynthesis is well documented (SHANKER et al., 2005; VERNAY et al., 2007), whilst for aquatic plants their potential in removing metals ions from aquatic environments has received more attention (LU et al., 2004).
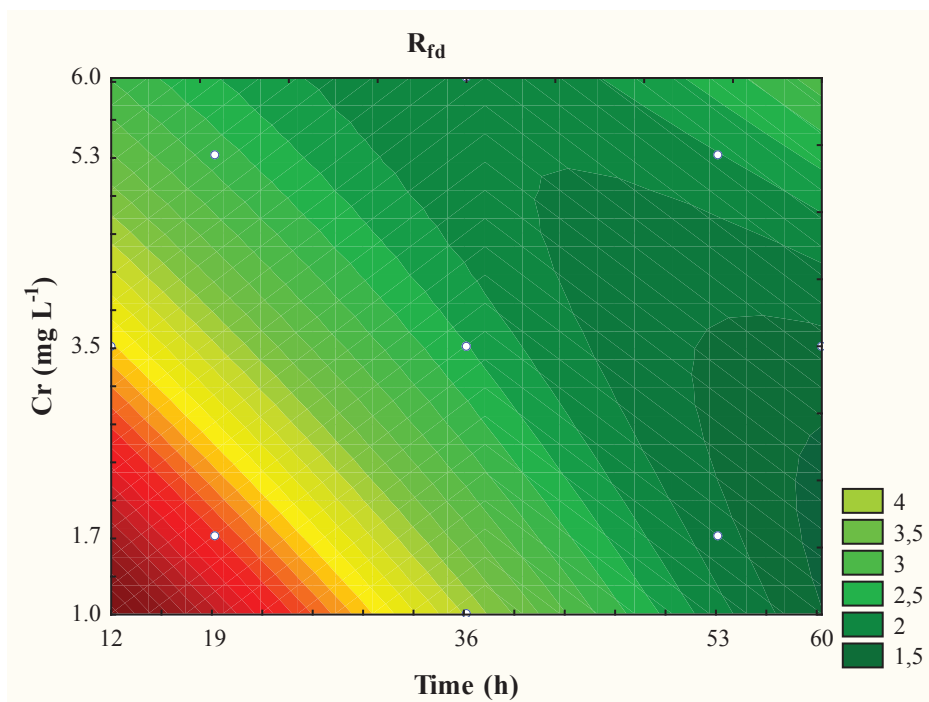
Figure 11. Contour surface plot of Vitality index (R_{fd}) as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

The disorganization of the chloroplast ultrastructure and inhibition of electron transport processes due to Cr and a desviation of electrons from the electron-donating side of PSI to Cr (VI) is a possible explanation for the Cr-induced decrease in photosynthetic rate (SHANKER et al., 2005). Paiva et al. (2009) related that there is little evidence of a correlation between PSII activity, CO_2 assimilation and heavy metal accumulation under conditions of excess Cr in aquatic plants. Other aspect related by Sinha et al. (2005) says that to tolerance capacity of the *P. stratiotes* to the metal depends on the balance of the factors favoring stress and factors reducing stress. And, according to Paiva et al. (2009) there is a group of plant species (termed hyperaccumulators) have the ability to accumulate non-essencial metals, such as Cr, and apparently do not show damage.

In general, it was possible to observe that the photosynthetic parameters of *P. strati*otes were affected by the stresse induced by chromium in a different way. The yield and R_{fd} were more sensitive to the higher chromium concentrations and to the longer time exposition. The non-photochemical dissipation has increased in the higher chromium concentrations, yet in the initial periods. These parameters present themselves as more sensitive, but more studies are necessary in order to understand the relations between the chlorophyll fluorescence parameters and the stressed caused by chromium. Despite that, it is possible to consider the proposal of using those parameters as a rapid and sensitive tool to the ecotoxicologic bioassay as promising.

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CAPÍTULO 7. ASSESSMENT OF CHROMIUM-INDUCED OXIDATIVE STRESS BY THE CENTRAL COMPOSITE DESIGN (CCD) AND RESPONSE SURFACE METHODOLOGY (RSM) IN *PISTIA STRATIOTES* L.

ABSTRACT

The central composite design was employed to investigate the effect of different nominal concentrations of chromium (VI) (1.0, 1.7, 3.5, 5.3 and 6.0 mg l^{-1}) applied for 12, 19, 36, 53 and 60h in *P. stratiotes* L. on the bioaccumulation, the chlorophyll content and the induction of oxidative stress. In roots, the bioaccumulation was higher than the leaves. The decrease in the contents of chlorophyll *a* and *b* was observed only in the more elevated concentrations of chromium and exposure time. The lipid peroxidation as well as activity of catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11) and glutathione reductase (GR; EC 1.6.4.2) showed increased, but there was variance in the exposure time and chromium concentrations when that occurred. An analysis by non-denaturing PAGE followed by staining for enzyme activity, revealed four GR isoenzymes in roots and five GR isoenzymes in leaves, the superoxide dismutase (SOD; EC 1.15.1.1) revealed four isoenzimes in roots and six isoenzymes in leaves. The results demonstrated that CCD and RSM represent tools suitable to assess the oxidative stress induced by chromium. *P. stratiotes* showed capacity to tolerate higher chromium concentrations. The rapid response of some of these enzymes could represent the use of this species as bioindicator sensitive of environment polluted with chromium (VI).

Key-words: bioaccumulation, chlorophyll, enzymes, experimental design, lipid peroxidation, metal, water lettuce.

<div style="text-align:center">RESUMO</div>

Avaliação do estresse oxidativo induzido por cromo através do Delineamento Composto Central (DCC) e Metodologia de Superfície de Resposta (MSR) em *Pistia stratiotes* L.

O Desenho Composto Central (CCD) foi empregado para investigar o efeito de diferentes concentrações nominais de cromo (VI) (1,0; 1,7; 3,5; 5,3 and 6,0 mg l^{-1}) aplicado por 12, 19, 36, 53 and 60h em *P. stratiotes* L. na bioacumulação, no conteúdo de clorofila e na indução de estresse oxidativo. A bioacumulação observada nas raízes foi maior do que nas folhas. O decréscimo no conteúdo de clorofila *a* e *b* foi observado apenas nas concentrações mais elevadas de cromo e nos tempos de exposição mais prolongados. A peroxidação de lipídios bem como a atividade da catalase (CAT; EC 1.11.1.6), ascorbato peroxidase (APX; EC 1.11.1.11) e da glutationa redutase (GR; EC 1.6.4.2) mostraram aumento, mas houve variância nos tempos de exposição e nas concentrações de cromo no qual estes aumentos ocorreram. Análises em gel de eletroforese não-desnaturante seguido de revelação para análise da atividade enzimática revelaram: quatro isoenzimas de GR nas raízes e cinco isoenzimas de GR nas folhas, para a superóxido dismutase (SOD; EC 1.15.1.1) quatro isoenzimas foram reveladas na raiz e seis isoenzimas nas folhas. Os resultados demonstraram que o DCC e a MSR representam ferramentas desejáveis para avaliar o estresse oxidativo induzido por cromo e *P. stratiotes* mostrou capacidade de tolerar concentrações mais altas de cromo. A rápida resposta destas enzimas poderia sugerir o uso desta espécie como uma bioindicadora sensível de ambientes poluídos com cromo (VI).

Palavras-chave: alface d'água, bioacumulação, clorofila, desenho experimental, enzimas, metal, peroxidação de lipídios.

Chromium and its compounds have multifarious industrial uses. Hexavalent chromium compounds are used in industry for metal plating, cooling tower water treatment, hide tanning and, until recently, wood preservation. These anthropogenic activities have led to the widespread contamination the Cr shows in the environment and have increased its bioavailability and biomobility (SHANKER et al., 2005; CHOO et al., 2007).

Chromium compounds are highly toxic to plants and are detrimental to their growth and development (SHANKER et al., 2005) and can be causing alteration in the production of pigments (e.g., chlorophyll, anthocyanin) which are involved in the life sustenance of plants (BOONYAPOOKANA et al., 2002; CHOO et al., 2007; GANESH et al., 2008) or increased production of metabolites (e.g., gluthathione, ascorbic acid) as a direct response to Cr stress which may cause damage to the plants (SHANKER et al., 2003). The reaction of Cr with biological reductants produces short- or long-lived Cr intermediates of different valency states that in turn react with hydrogen peroxide to generate hydroxyl radical (STOHS; BAGCHI, 1995). It has now been invariably demonstrated that oxidative mechanisms are involved in the toxicity of metal ions in plants. Similar to other stresses, plant's response to heavy metals result in changes in the levels of antioxidants and antioxidative enzymes to detoxify the reactive oxygen species (ROS) (PANDEY et al., 2005). The well developed defense system comprising enzymes are namely superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), ascorbate peroxidase (APX), as well as non-enzymatic compounds (MALLICK; MOHN, 2000).

Thus, the knowledge of how plants cope with metal-induced oxidative stress is of considerable importance in understanding the metal tolerance mechanisms evolved by plants.

Some aquatic macrophytes can accumulate considerable amount of heavy metals in their tissues (MONFÉRRAN et al., 2009). Despite the intensive work on aquatic macrophytes for their potential use in phytoremediation, the relative contribution of the diverse mechanisms leading to metal detoxification and tolerance, as well as the interspecific difference in defense strategies, have been given scanty consideration so far (SANITÀ DI TOPPI et al., 2007).

Pistia stratiotes is a perennial aquatic macrophytes spread all over the world and this species carry out their entire life cycle free-floating on the water surface, and only the root system is completely submerged (PRASAD et al., 2001). The species take up metals from water, producing an internal concentration several fold greater than their surroundings and showing much higher metal-accumulating capacity (MAINE et al., 2001, PRASAD et al., 2001). Considering thus ability of *Pistia stratiotes* in incorporate metal, is used for phytoremediation of wastewater or natural water bodies polluted with heavy metals (MAINE et al., 2001, PRASAD et al., 2001, ODJEGBA; FASIDI, 2004, SUÑE et al., 2007). The species exhibit different response patterns of to Ag, Cd, Cr, Cu, Hg, Ni, Pb and Zn, and presented distinct levels of growth inhibition and biomass production, with almost all the elements being accumulated at high concentrations in the root systems (ODJEGBA; FASIDI, 2004).

The use of Central Composite Design (CCD) and Response Surface Methodology (RSM) in aquatic toxicology is recent and has presented promising results, herewith and considering this whole background of responses induced under chromium stress, the aims of this work was (1) to evaluate the effect of chromium on the bioaccumulation, lipid peroxidation and the induction of oxidative stress using this methodology; and (2) to evaluate the use of *P. stratiotes* as bioindicator of metal pollution, as well as its potential use for bioremediation of environment polluted with heavy metals.

Plant material and treatment conditions

Plants of *Pistia stratiotes* L. were obtained from São Paulo littoral and maintained at the Laboratory of Ecotoxicology and Ecophysiology of Aquatic Organisms of the Water Resources and Applied Ecology Center (CRHEA) of the University of São Paulo (São Paulo State, Brazil). The macrophytes were cultivated in containers (1000 L) with tap water and fertilizer KNP (4:14:8). The solution of cultivate was maintained at pH 7±0.5 by titration with dilute HCl or NaOH.

Plants with similar size and growth stage were selected, washed with in a running tap water and acclimated for two days in laboratory in nutrient solution containing (mM): 1.25 $Ca(NO_3)_2.4H_2O$, 1.5 KNO_3, 0.5, KH_2PO_4, 0.5 $MgSO_4.7H_2O$, 0.05 H_3BO_3, 0.01 $MnCl_2.4H_2O$, 0.30 $CuSO_4.5H_2O$, 0.20 Na_2MoO_{24} and 0.5 NaFeEDTA(10%Fe) (ODJEGBA; FASIDI, 2004).

Tests were performed with one plant in 1 liter nutrient solution submitted to different nominal concentrations of chromium (1.0-6.0 mg Cr L^{-1}) supplied as standard chromium (Merk), and exposure time (12, 19, 36, 53 and 60 h) (Table 1). Plants in nutrient solution without chromium served as a control. Both the control and the treated solutions were maintained at pH 5.5 to garanted metal in solution and available for absorption by plant roots (ODJEGBA; FASIDI, 2004). The macrophytes during the test were maintained at 26±2°C and under a constant irradiance of 7.000 lux provided by cool-white fluorescent lamps.

Chromium quantification

The leaves and roots *P. stratiotes* were washed with mili-Q water, and oven dried for 48 h at 60 ºC. The preparation of samples for chromium estimation was carried out by acid digestion (HNO_3 and H_2O_2) at 120 °C and then diluting them with mili-Q water, and every analytical procedure was accompanied by an analytical blank (APHA, 1995). The measured concentration of metal, expressed as mg Cr mg^{-1} dry weight. All the samples were analyzed in triplicates by graphite-furnace atomic absorption spectrometry (Varian AA 220). The recoveries of metals from the plant tissues were found to be more than 95.5%, the detection limit was 0.47 µg l^{-1} and the quantification limit was 1.55 µg l^{-1}.

Lipid peroxidation

The level of lipid peroxidation in plant roots and leaves was determined by estimation of the malondialdehyde (MDA) content based on the method of Heath and Packer (1968). Thiobarbituric acid-reacting (TBARS) substances representing the lipid peroxidation product were extracted by homogenization of leaves and roots from *P. stratiotes* (300 mg) with 20% (w/v) insoluble polyvinylpyrrolidone (PVPP) and 1.3 ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10 000g for 5 min., and was added 1 ml 0.5% TBA in 25% TCA at 250 µl of the supernatant. The mixture was heated at 95 °C for 30 min and the reaction was stopped by quickly transferring the mixture to an ice bath. The absorbance of the TBARS was determined spectrophotometrically at 535 nm. Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm, and the concentration of TBARS was calculated using the absorbance coefficient 1.55 x 10^{-5} mol^{-1} cm^{-1}.

Assay of antioxidant enzymes

All biochemical analyses were performed at 4 °C unless stated otherwise. The samples of leaves and roots from *P. stratiotes* were homogenized (300 mg FW) with potassium phosphate buffer (pH 7.5) containing 1mM ethylenediaminetetraacetic acid (EDTA), 3 mM $_{DL}$-dithiothreitol and 5 % (w/v) insoluble PVPP. Homogenate was centrifuged at 10 000g for 30 min and the supernatant was stored in aliquots at -80 °C and was used to measure the activities of CAT, APX and GR. Proteins content was measured according to Bradford (1976) using serum albumin as the standard protein.

The total activity of CAT (EC 1.11.1.6) in leaves and the roots from *P. stratiotes* was determined spectrophotometrically by monitoring the degradation of H_2O_2 at 240 nm over 1 min against a plant extract-free blank (AZEVEDO et al., 1998). APX (EC 1.11.1.11) total activity was measured in leaves and the roots by the method of Nakano and Asada (1981), by monitoring the rate of ascorbate oxidation at 290 nm at 30 °C. The activity was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1}. Total GR (1.6.4.2) activity leaves and the roots were determined spectrophotometrically as described by Azevedo et al. (1998). The reduction of GSSG (Oxidized Glutathione) was followed by monitoring the increase in absorbance at 412 nm over 2 min.

Polyacrylamide gel electrophoresis (PAGE)

SOD and GR were evaluated by electrophoretic analysis under non-denaturing condition in 12 % polyacylamide gels as described by Medici et al. (2004).

GR activity in native PAGE gels was determined as described by Lee and Lee (2000) with modifications as described by Medici et al. (2004). SOD activity was determined as described by Beauchamp and Fridovich (1971) and modified by Azevedo et al. (1998).

Photosynthetic pigments estimation

Leaves discs of *P. stratiotes* presented surface area ca. 200 mm^2. These discs were extracted with 2 ml aqueous acetone 80% in a mortar with pestle. The homogenate, combined with a further three washings of the pestle and mortar (each of 1.5 mL) with the same solvent, was centrifuged at 2500 rpm for 10 min. The pellet was then extracted with a further 1 mL of solvent and the pooled supernatants adjusted to a final volume of 8 ml (PORRA et al., 1989). The absorbance at the major absorption peak of chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) and Chlorophyll total (Chl *a* + Chl *b*) was measured in Spectrophotometer and the concentrations in μg l^{-1} were then calculated according to Porra et al. (1989).

Statistical analysis

Toxicity tests with *P. stratiotes* were conducted in 11 experiments for the study of two parameters (see matrix in table 1). The model studied is a 2^2 factorial experimental design. We selected time of exposure (X1) and chromium concentration (X2) as independent variables. The experimental results of the response surface methodology were fitted with a second-order polynomial equation (1) by a multiple regression technique

$$Y = b_0 + b_1 \, x_1 + b_2 \, x_2 + b_{11} \, x_1^{\,2} + b_{22} \, x_2^{\,2} + b_{12} \, x_1 \, x_2 + \varepsilon \qquad (1)$$

Y is the predicted response, b_0, b_1, b_2, b_{11}, b_{22}, b_{12} are constant coefficients, and x_1, x_2 are the coded independent variables or factors.

The test factors were coded according to the following regression equation (2):

$$x_i = \left(\frac{X_i - X_0}{\Delta X_i} \right) \qquad (2)$$

where x_i is the *coded* value and X_i is the *actual* value of the *i*th independent variable, X_0 is the actual value at the center point, and ΔX_i is the step change value. Statistic 7.0 software (Statsoft, USA) was used for regression and graphical analysis of the data.

Table 1 Process variables used in the CCD showing the treatment combinations between chromium concentration and exposure time in *P. stratiotes*.

Runs	Coded setting levels		Actual levels	
	x1= time; x2= [Cr]		X1= time (h); X2= [Cr] (mg l^{-1})	
	x1	x2	X1	X2
1	-1	-1	19	1.7
2	-1	1	19	5.3
3	1	- 1	53	1.7
4	1	1	53	5.3
5	0	0	36	3.5
6	0	0	36	3.5
7	0	0	36	3.5
8	0	- 1.41	36	1.0
9	0	1.41	36	6.0
10	-1.41	0	12	3.5
11	1.41	0	60	3.5

The percentage of variation explained by the the second-order model equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by an *F*-test. The significance of the regression coefficients was tested by a *t*-test and *p*-values.

7.3. RESULTS AND DISCUSSION

Stress parameter and toxic element concentration

The results obtained from 2^2 factorial designs experimental to metal concentration and chlorophyll content in *P. stratiotes* are shown in Table 2. The analyses of metals concentration in roots and leaves demonstrated that accumulation of chromium by the roots was increased as compared with the leaves and, influenced by the interaction two factors: exposure time and chromium concentration. A response surface analysis of the chromium concentration in roots and leaves was performed to investigate the removal of these metals by *P. stratiotes*. The coefficient of regression (75 and 98%, in roots and leaves, respectively) verified the adequacy of using the regression model for describing the contour surface with 95% of confidence (Table 3 and Figures 1 and 2).

Table 2 The mean experimental design responses with the results obtained from the activity of enzymes catalase (CAT), ascorbate peroxidase (APX) and Glutathione Reductase (GR) in $\mu mol\ min^{-1}\ mg\ protein^{-1}$, the estimative of lipid peroxidation through TBARS content in $M\ g^{-1}$, the chromium concentration in $mg\ L^{-1}\ dry\ wt^{-1}$ in roots and $\mu g\ L^{-1}\ dry\ wt^{-1}$ in leaves, and the chlorophyll content in $\mu g\ L^{-1}\ f\ wt^{-1}$.

| Runs	CAT	TBARS	[Cr]	CAT	APX	GR	TBARS	[Cr]	Chl a	Chl b
		Roots					Leaves			
1	101.13	1.60	81.20	74.00	81.30	1.77	2.14	8.87	188.75	288.82
2	46.61	3.09	86.34	64.50	66.58	2.51	1.48	16.71	197.86	361.73
3	92.90	2.04	87.45	98.88	61.68	1.11	2.08	15.44	146.29	263.53
4	142.20	1.88	114.03	47.46	62.38	0.17	1.15	56.12	214.85	392.51
5	62.57	3.06	92.15	31.63	57.10	5.11	1.69	18.74	185.39	291.12
6	64.08	2.91	91.29	33.07	43.02	3.26	1.39	20.43	186.09	283.28
7	61.07	3.20	90.44	34.51	50.06	4.18	1.99	17.05	187.27	298.96
8	65.65	2.81	44.38	42.65	76.87	1.03	2.56	8.86	201.62	369.61
9	93.44	3.08	60.36	15.73	31.93	1.60	1.74	38.73	168.69	308.16
10	80.97	2.81	147.08	95.91	161.78	5.64	2.05	4.80	193.04	311.30
11	103.67	2.09	56.44	13.06	38.31	0.61	2.00	30.03	228.10	405.05
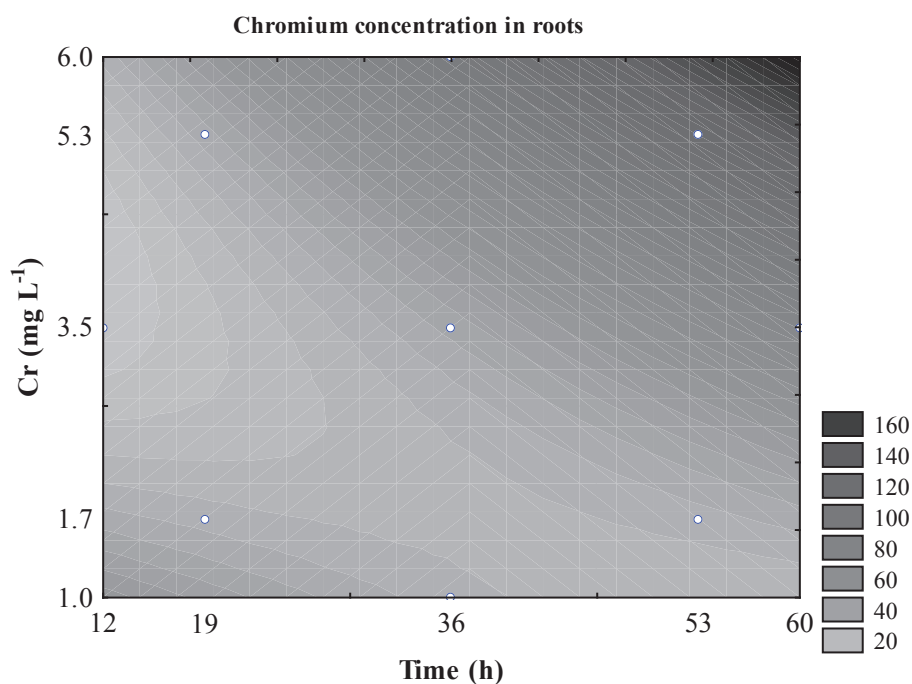
Figure 1. Contour surface plot of bioaccumulation in roots as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.
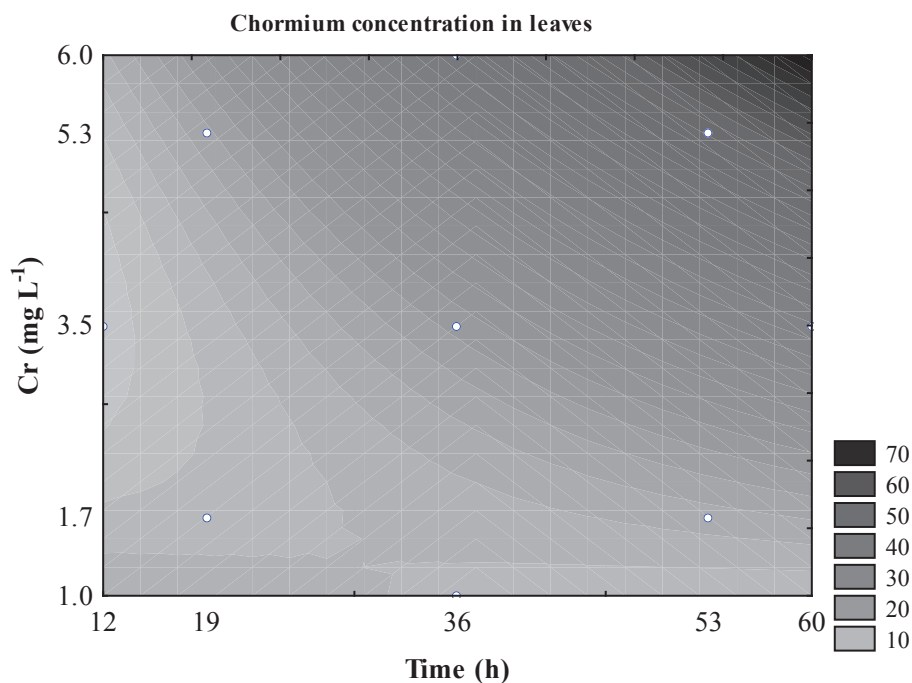

Figure 2. Contour surface plot of bioaccumulation in leaves as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

In this model, time and chromium linear were significantly different in roots (Table 3). In leaves, exposure time and chromium concentration linear were significantly different and chromium quadratic and the interaction between them (time x chromium) (Table 3), which indicates that they act as limiting factors and even small variations in their values will alter the bioaccumulation in leaves to a considerable extent.

The response surface analysis of chlorophyll content was performed and the elevated coefficient of regression 92% to Chl a and 88% to Chl b verified the adequacy of using the regression model for describing the contour surface (Figures 3 and 4). The model of chlorophyll a content represents that it was significantly affected by exposure time linear and quadratic ($p < 0.05$), i.e. the longer the exposure time to metal, more pronounced the response will and for chlorophyll b the chromium concentration (linear term) and the interaction between two factors (exposure time and metal concentration) were significantly different ($p < 0.05$).

Table 3. Statistical analysis of different parameters analyzed by experimental design. The activity of enzymes catalase (CAT) and Glutathione Reductase (GR) are in µmol min^{-1} mg protein^{-1}, the estimative of lipid peroxidation through TBARS content in mM g^{-1} fr. wt., the chormium concentration [Cr] in µg L^{-1}, and the chlorophyll content in µg L^{-1} g^{-1} fr. wt.

Terms	Coefficient estimate (± Standard error)									
	Roots			Leaves						
	CAT	TBARS	[Cr]	CAT	APX	GR	TBARS	[Cr]	Chl a	Chl b
b_0	***62.535 (± 7.4)***	***3060.529 (± 223.9)***	***9.133 (± 1.2)***	***33.006 (± 8.0)***	***50.097 (± 15.1)***	4.187 (± 0.6)	***1690.464 (± 182.2)***	***18.724 (± 1.8)***	***186.251 (± 5.1)***	***291.120 (± 13.5)***
b_1	***14.967 (±4.5)***	-225.410 (± 137.3)	***1.855 (± 0.7)***	-9.369 (± 9.8)	***-24.813 (± 9.3)***	***-1.266 (± 0.4)***	-56.359 (± 111.8)	***10.225 (± 1.1)***	***-9.004 (± 3.1)***	-10.176 (± 8.3)
b_2	***17.405 (± 5.4)***	***-444.148 (± 163.9)***	0.076 (± 0.8)	***55.476 (±11.8)***	22.760 (± 11.1)	***-0.739 (± 0.4)***	72.162 (± 133.4)	0.2641 (± 1.3)	***-3.279 (± 3.7)***	18.412 (± 9.9)
b_{11}	4.258 (± 4.5)	213.937 (± 137.3)	***1.931 (± 0.7)***	***-24.794 (± 9.8)***	-9.702 (± 9.3)	0.075 (± 0.4)	***-344.621 (± 111.8)***	***11.363 (± 1.1)***	***15.907 (± 5.1)***	***41.808 (± 8.3)***
b_{22}	10.979 (± 5.5)	-194.853 (± 163.9)	0.420 (± 0.8)	4.485 (± 11.8)	-0.200 (± 11.1)	***-1.651 (± 0.4)***	135.607 (± 133.4)	***3.472 (± 1.3)***	9.429 (± 3.7)	***28.056 (± 9.9)***
b_{12}	***25.957 (± 6.4)***	-415.028 (± 193.9)	0.536 (± 0.9)	-20.966 (± 13.9)	3.855 (± 13.1)	-0.418 (± 0.5)	-68.203 (± 157.8)	***8.207 (± 1.6)***	***14.066 (± 4.4)***	14.017 (± 11.7)
R^2	0.88896	0.77500	0.74949	0.86801	0.72162	0.84609	0.68841	0.97776	0.91565	0.8827
F-value	18.68	31.00	11.97	26.30	23.33	21.99	19.88	65.94	25.33	30.10
F stat table	4.35	5.12	4.46	4.46	5.12	4.46	5.12	4.53	4.35	4.46
F cal/Ftab	4.29	6.05	2.68	5.89	4.56	4.93	3.88	14.56	5.80	6.75

Values of terms in bold and italic are significantly different at $P < 0.05$.

Chlorophyll *a* content

Figure 3. Contour surface plot of chlorophyll *a* content as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

Chlorophyll *b* content
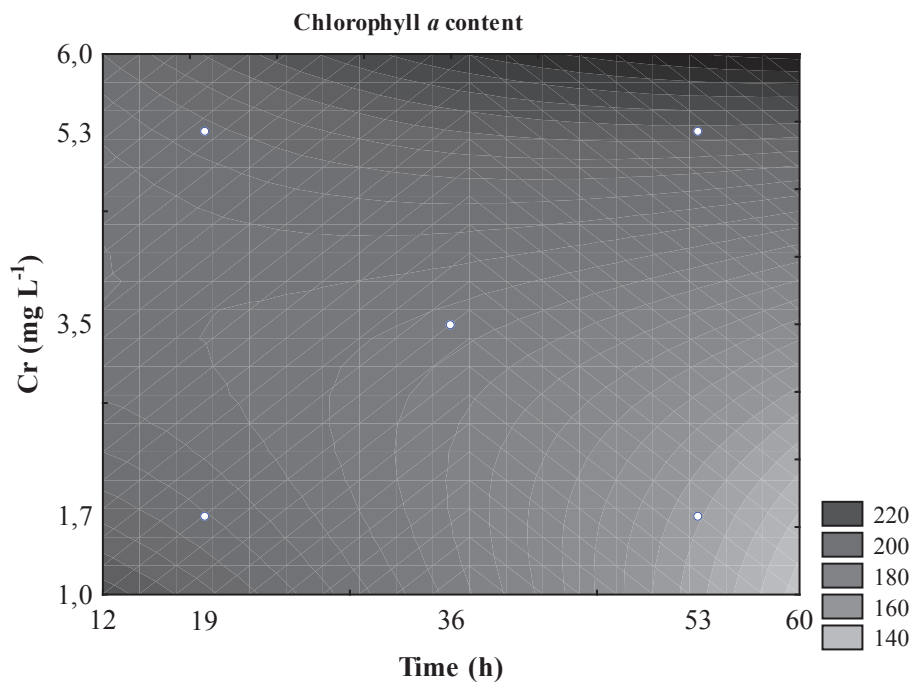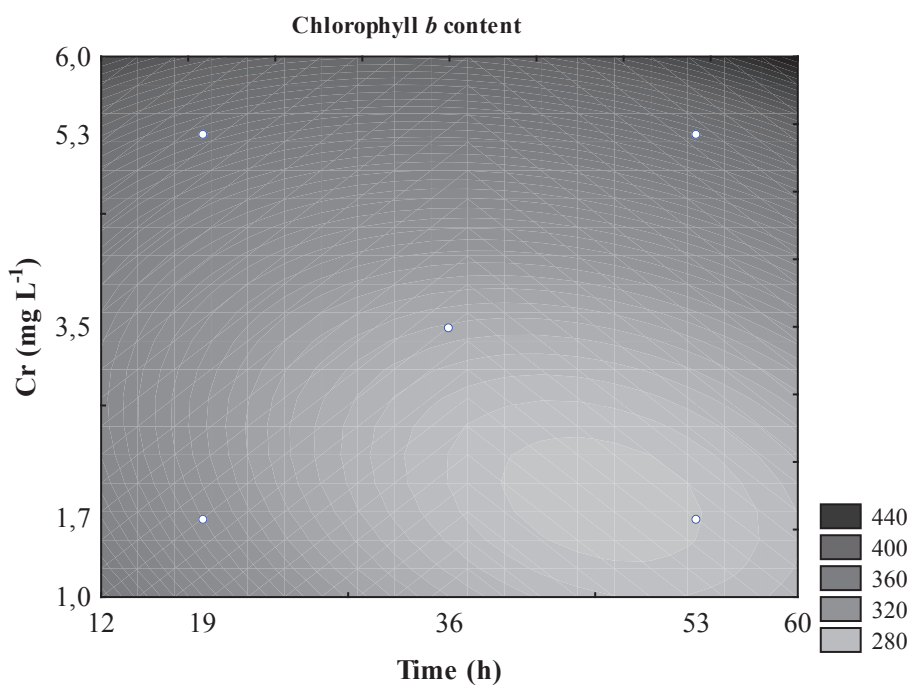
Figure 4. Contour surface plot of chlorophyll *b* content as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

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In general, there was increase in Chl *a* content as compared to control plants. In the center point there was alteration in the Chl *a* content, but the content decreased in the more elevated exposure time (53 and 60h). On the other hand, the chlorophyll b content was significantly affected by chromium concentration linear and quadratic where this factor could be limiting to the production of this pigment. The increase of Chl *b* content in relation to control plants occurred almost every exposure time (12 to 36h) and decrease in the more elevated exposure time (53 and 60h), except for run 4 (53h and 5.3 mg Cr l^{-1}) where there was an elevation in the content of Chl *b*. These results can be observed in the contour surface and contrary in part to what was related by literature. Significant increased in chlorophyll and carotenoids contents were seen in mustard plants exposed to 2.0 mg l^{-1} Cr (VI) (PANDEY et al., 2005). Chlorophyll content also was high in tolerant calluses in terms of survival under high Cr concentration in a study of Cr and Ni tolerance in *Echinochloa colona* (SAMANTARAY et al., 2001). Chlorophyll content decrease as a marked effect of various concentrations of different Cr compounds [Cr (III) and Cr (VI)] in *Triticum aestivum* (SHARMA; SHARMA, 1993). Although, both increase and decrease in the chlorophyll content has been reported in different plant species exposed to Cr (VI) (SHARMA; SHARMA, 1993; SAMANTARAY et al., 2001), in general, Cr (VI) adversely affected the chlorophyll content and produces a decrease in photosynthetic pigments in various aquatic plants vascular (SHANKER et al., 2005; SINGH; SINHA, 2005; CHOO et al., 2007; GANESH et al., 2008). Maine et al., (2004) related a light decline in chlorophyll content in *P. stratiotes* for Cr concentration in water over 1 mg l^{-1}. The reduction in the chlorophyll content has been reported in *Pistia stratiotes*, *Hydrilla verticillata*, *Ceratophyllum demersum* plants exposed to Cr, Cu and Pb, respectively (SINHA et al., 2005; MISHRA et al., 2006; SRIVASTAVA et al., 2006).

Lipid peroxidation induced by metals profoundly alters the structure of membranes and consequently modifies their enzymatic and transport activities (SINHA et al., 2005). The results obtained from design experimental to TBARS content which indicate the occurrence of lipid peroxidation in *P. stratiotes* also are shown in Table 2, and the analysis of TBARS content in roots and leaves demonstrated distinct comportment. The response surface analysis to TBARS content was performed, indicating that 78% and 69% of the variability on the data could be explained by the regression model describing the contour surface (Figures 5 and 6). The statistical analysis was significantly affected by exposure time linear in roots and exposure chromium concentration in leaves ($p < 0.05$) (Table 3). The lipid peroxidation estimated by TBARS content in roots was more intense above 3.5 mg Cr l^{-1} which can be observed in the presence of curvature in the regions of interest in the contour surface (Figure 5).
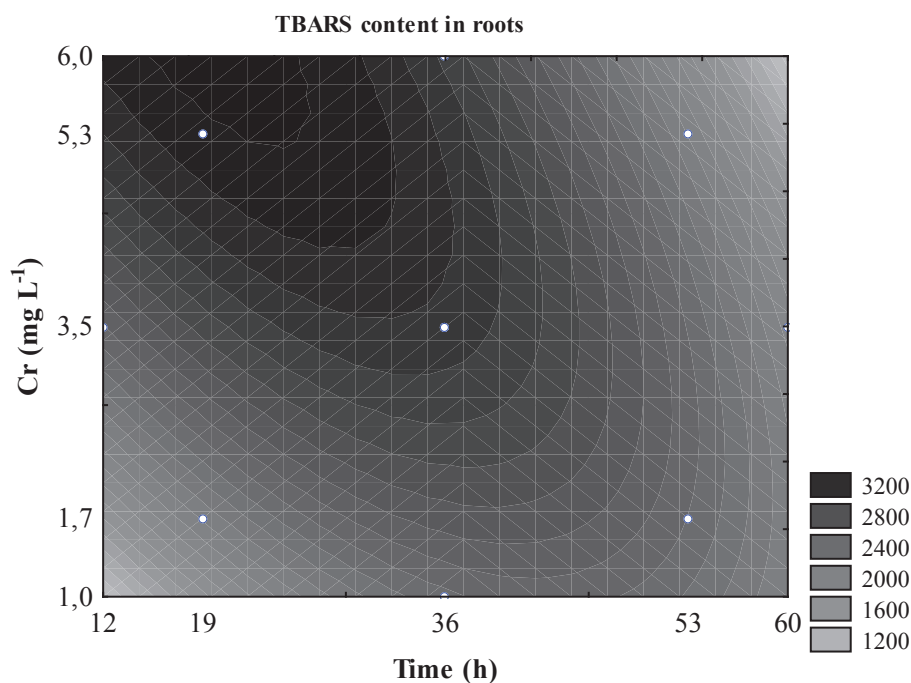

Figure 5. Contour surface plot of TBARS content in roots as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

In the leaves, the response observed was that the lipid peroxidation was more elevated in the smaller Cr concentration (1.0 and 1.7 mg l^{-1}). The analysis of contour surface of lipid peroxidation (Figure 6) showed that the obscure region (where lipid peroxidation is higher) coincides with the region where the Chl *a* content is smaller (lighter region) according to mathematical models performed for two parameters. According to Somashekaraiah et al. (1992) the reduction in chlorophyll content in *P. stratiotes* could be attributed due the peroxidation of chloroplast membranes due to increased production of free radicals, inhibition of δ-aminolaevulinic acid dehydratase activity, or by increased chlorophyllase activity. Another aspect is the interaction of metal to -SH group of enzymes of chlorophyll biosynthesis, as well as lipid peroxidation mediated degradation, which are in agreement with other reports in aquatic plants (SINHA et al., 2002; ASLAN et al., 2003).
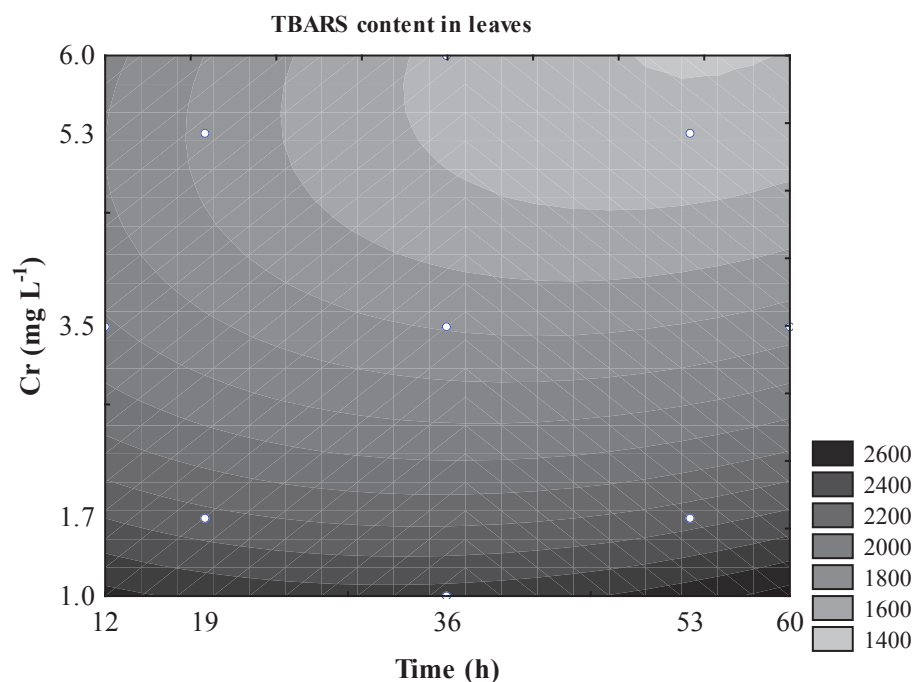

Figure 6. Contour surface plot of TBARS content in leaves as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

Likewise, in this study, chromium induced oxidative stress in *P. stratiotes* was evident from the increased lipid peroxidation in its roots and leaves, which is in agreement with the other studies carried out in hydroponics systems (GALLEGO et al., 1996; SINHA et al., 2005).

Antioxidant enzymes activity

Heavy metal toxicity due its accumulation is considered to induce the production of ROS and may result in significant damage to cellular constituents (SINHA et al., 2005). The probable damage can be eliminated or reduced through the plant defense system composed by enzymatic and non enzymatic mechanisms. The enzyme activity parameters are the most sensitive ones in evaluating the effects of stress on plant system (GANESH et al., 2008). The changes in enzymatic activities of CAT, APX and GR obtained by experimental design of *P. stratiotes* being exposed to Cr (VI), are shown in Table 2. The activity of CAT in roots was bigger when compared to the activity of control plants in different times: 19, 53 and 60 hours of exposure to chromium. In the intermediate time (36h) the activity of CAT in roots of control plants was greater, but the enzymatic activity was increased with the elevation the chromium concentration (Table 2). In leaves of macrophytes, the enzymatic activity of CAT was also greater and decreased in relation to control plants, and the response was less intense that in roots.

The high coefficient of determination, 89% in roots and 87% in leaves, demonstrated that the variation in CAT activity submitted to different chromium concentration could be explained by the model of regression performed (Table 3). The statistical analysis of this enzyme was significantly affected by exposure time linear and quadratic ($p < 0.05$) and by

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interaction between exposure time and chromium concentration in roots. The exposure time quadratic and chromium concentration linear were significantly different ($p < 0.05$) in CAT activity in leaves (Table 3). Thus, as exposure time (quadratic term) was significant, the CAT activity will be determined by this parameter, but with influences on the chromium concentration, because there was interaction between him. The contour surface developed by model of regression in figures 7 and 8, presented positive values to coefficients of the activity of CAT in roots and some negative coefficients in leaves, implying the presence of curvature form to be either concave upward or downward depending on the exposure time and chromium concentration.

The mean values for specific CAT activity were circa 80 in roots and 50 in leaves, and these values reveal a very high increase in relation to literature. Ganesh et al. (2008) worked with *P. stratiotes* submitted to Cr (VI), reported a gradual decrease of the activity of CAT varying from 0 to 3.0 units min^{-1} mg^{-1} protein. Values still smaller (activity of CAT < 1.0), but with increased activity, were obtained by Odjegba and Fasidi (2007) working with the same species and various heavy metals, including chromium. The CAT activity in *P. stratiotes* under Cd stress showed in roots activity below the detection limits and in leaves was stimulated by 25µM Cd and completely abolished in 100µM Cd (SANIÀ DI TOPPI et al., 2007). Teisseire and Guy (2000) reported an enhancement of CAT activity in *Lemna minor* fronds exposed for 24h to a range of 0 to 10 µM of Cu.
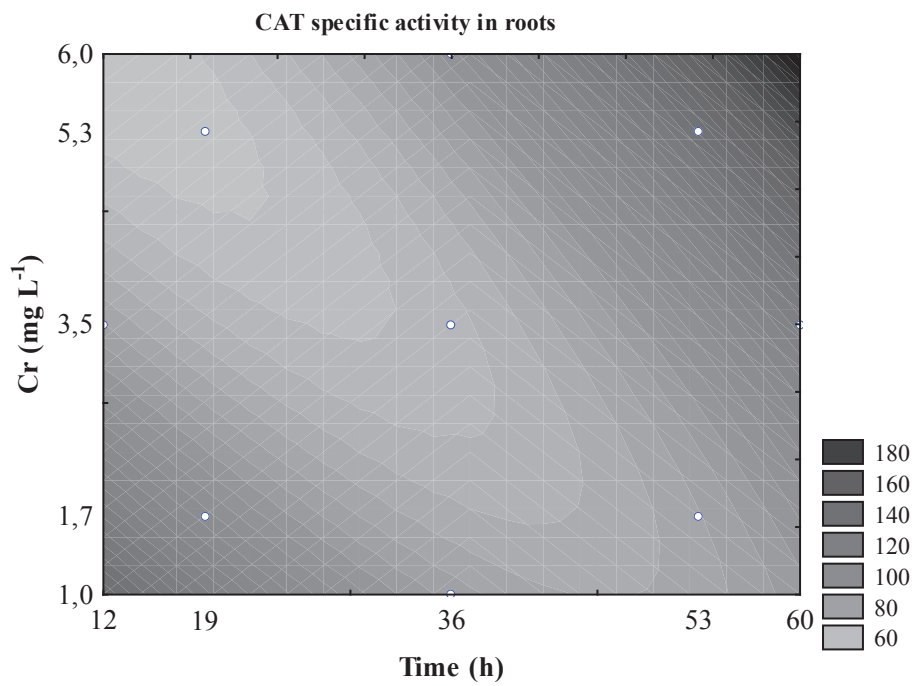
Figure 7. Contour surface plot of CAT in roots as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

Figure 8. Contour surface plot of CAT in leaves as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

Other studies have reported that a decrease in CAT activity in plants under Cu stress might be due to the replacement of total or partial Fe from the active sites (AGARWALA et al., 1977; LUNA et al., 1994; SRIVASTAVA et al. 2006). The stressed plant cells simultaneously enhance synthesis of antioxidant compounds (ascorbic acid, cysteine, NP-SH) to counter with the induced stress situation. Ascorbic acid serves as a substrate for APX, a key enzyme of the ascorbate cycle and is converted to dehydroascorbate (DHA) during elimination of peroxides (SINHA et al., 2005). The results obtained from design experimental to activity of APX in leaves of *P. stratiotes* are shown in Table 2. A response surface analysis of the chromium concentration in roots and leaves was performed, but the differences were significant only for the leaves. The coefficient of determination was 72% ($p < 0.05$), which verified the adequacy of using the regression model for describing the contour surface (Figure 9). In this model, only exposure time linear was significant in leaves (Table 3). Whilst not significant, the APX activity in roots showed higher than control plants with 19, 53 and 60h of exposure to chromium, varying from 30 to 200 units min^{-1} mg^{-1} protein (data not shown). The activity of APX in leaves was bigger in the lower exposure time (12 and 19h), where more intense activity was observed as compared to control plants, varying from 30 to 160 units min^{-1} mg^{-1} protein. Sinha et al. (2005) observed increase in the APX activity in plants of *P. stratiotes* with increasing externally supplied Cr (VI) concentrations. Srivastava et al. (2006) reported increase at shorter durations in the activity of APX up to 1.5 mg Cu l^{-1} working with a weed aquatic *Hydrilla verticillata*. The induction of APX activity in other macrophytes also reported in *Bacopa monnieri* under Fe and Cu stress (KAMPFENKEL et al., 1995; GUPTA et al., 1999), *Ceratophyllum demersum* under Cu stress (DEVI; PRASAD, 1998) and *Vallisneria natans* under ammonia stress (WANG et al., 2008).
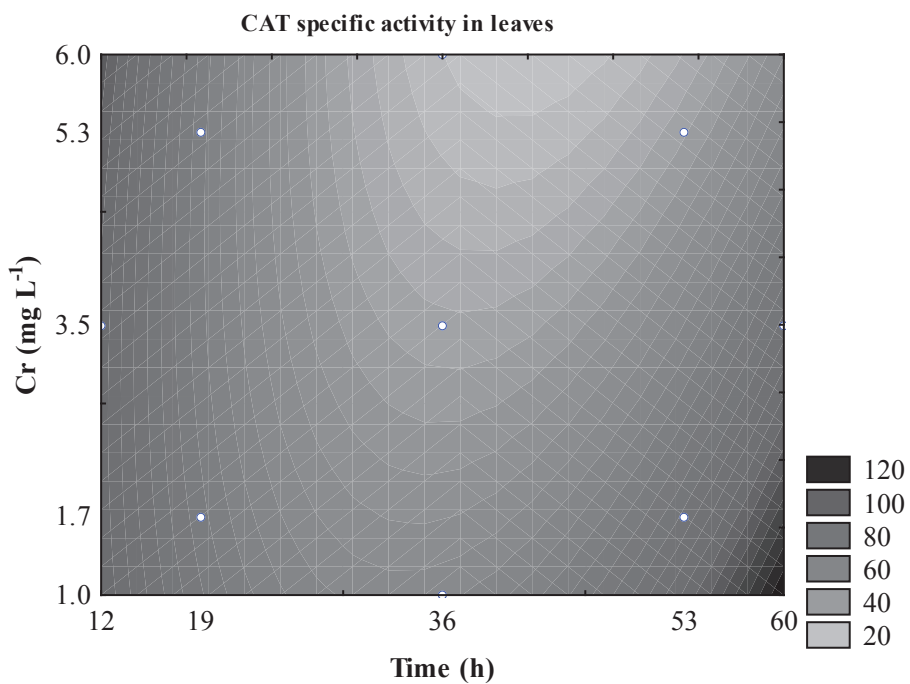
APX specific activity in leaves
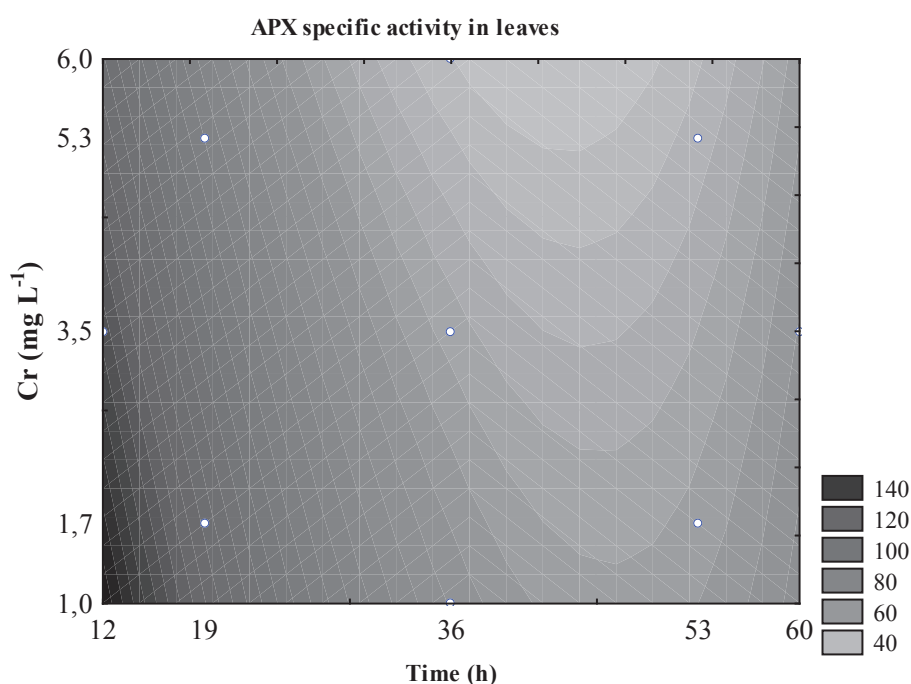
Figure 9. Contour surface plot of APX in leaves as a function of chromium concentration (1.0-6.0 mg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

The production of ROS in cells may cause an increase of peroxides levels, which can be metabolized by the glutathione cycle. The key function of GR within this cycle is to maintain the GSH/GSSG ratio playing a crucial role for the antioxidant system as well as for primary detoxication pathways (MISHRA et al., 2006). Similarly the analysis of APX, the response surface analysis of GR activity of the chromium concentration in roots and leaves was performed, but the differences were significant only for the leaves. The activity of GR was bigger than the control plants in almost all exposition time and chromium concentrations, varying from circa 1.0 to 9.0 units min^{-1} mg^{-1} protein (data not shown). The activity of GR variation in leaves was from 0.2 to 5.7 units min^{-1} mg^{-1} protein, and when compared to control plants was higher until intermediate exposure time (12, 19 and 36h).

The contour surface developed by model of regression in figure 3B, has presence of curvature concentrated in these regions (intermediate time) and chromium concentrations from 1.7 to 5.3 mg l^{-1}. 85 % of variability from the results can be explained by the model of regression performed ($p < 0.05$) where the time linear and quadratic and chromium quadratic were significantly different. These results reinforced that these factors act as determinants, stimulating the enzymatic activity of GR.

Sanità di Toppi et al. (2007) working with *P. stratiotes* and *Eichornia crassipes* under Cd stress, reported about complex relationships observed among treatments effects on the GSSH levels where the GR activity was remarkably stimulated by exposure to Cd in the leaves of both species. In the roots, instead, GR activity was increased in *P. stratiotes* and decreased in *E. crassipes*. Srivastava et al. (2006) working with *H. verticillata* suggest that exposure to copper concentrations beyond 1 μM for longer durations were toxic and thus would have disturbed its role. The role of GR is crucial for the maintenance of plant metabolism as GSH is not only required for the synthesis of phytochelatins and functioning of ascorbate-glutathione cycle but it also needed as reductant in many biochemical reactions. GR activity in roots staining revealed the presence of at least four GR isoenzymes in *P. stratiotes* (bands I-IV) (Figure 10A). There was increased activity of GR which exhibited band more intense in response at 3.5 mg Cr l^{-1} (lane 3) and less intense at 1.0 mg Cr l^{-1} (lane 2). The spectrophotometric analysis of GR activity showed that run 5 (7.7 μmol min^{-1}mg protein^{-1}) is twofold bigger than run 8 (3.4 μmol min^{-1}mg protein^{-1}). In leaves of *P. stratiotes*, activity of GR staining revealed the presence of at least five isoenzymes (bands I-V) (Figure 10B), with expression differential these isoenzymes in the different treatments. Bands III and V exhibit activity of two GR isoenzymes in all treatments with chromium, including control plants. Band I exhibits expression of one isoenzyme less intense in the control plants (lane 1) and in

the chromium concentration at 6.0 mg l^{-1} (lane 4). An isoenzyme of GR presented in band II stained more intense in response at 3.5 mg l^{-1} and extremely less intense in the other treatments. In contrast, band IV exhibits activity (more electropositive) only in response to 6.0 mg l^{-1} of chromium.

Amongst various enzymes involved in the abolishment of ROS, SOD can be considered as a key enzyme. SOD is the first enzyme in detoxifying processes, it converts O_2^- radicals to H_2O_2 (SINHA et al., 2005). In this work the activity of SOD will be present only by electrophoretic analysis. SOD activity in roots of *P. stratiotes* staining revealed at least four isoenzymes Mn-SODs (proof not shown) (bands I-IV) (Figure 11A). For bands II and III the isoenzyme of SOD exhibited increase in intensity in response to chromium treatment (more electropositive), but band II was less intense. Band I only expressed activity of first isoenzyme of SOD in response at 3.5 mg Cr l^{-1}. In leaves of *P. stratiotes*, SOD activity staining revealed at least six isoenzymes (bands I-VI) (Figure 11B). All SOD activity bands exhibited expression in response to chromium, but two (the more electropositive) revealed increase in response intensity to chromium treatment.

Chromium has been demonstrated to stimulate formation of free radicals and ROS (STOHS; BAGCHI, 1995). To scavenge ROS and to avoid oxidative damage, cells are normally protected by the operation of intricate antioxidant systems, comprising both enzymatic systems and non-enzymatic systems. Peroxidases (such as SOD, APX, GPX) are known to play a significant role in oxidative stress conditions and it has been shown that peroxidase activity can be used as a potential biomarker for sublethal metal toxicity in examined plant species (RADOTIC et al., 2000).

The majority of these enzymes presented response in the initial time after exposure to the metal that was the case for CAT, APX and GR. It is important to highlight that the response of APX was more intense in leaves than in roots, where the accumulation of metal was less intense. This aspect could be occurring because the accumulation of metal increased in a time-dependent manner (MONFERRÁN et al., 2009). The quickness of response and the sensibility of these enzymes to metal could be an indicative of *P. stratiotes* as bioindicator and proposed as a good biomonitor for the assessment of metal pollution in aquatic systems. Upadhyay and Panda (2009) reported that the rapid inducibility of those enzymes in *P. stratiotes* upon copper stress is useful and sensitive indicators of heavy metal toxicity (UPADHYAY; PANDA, 2009).

Figure 10. Activity staining for GR (Glutathione reductase) isolated from roots (A) and leaves (B) of *P. stratiotes* after 36h of exposition to chromium. Lane 1, control; Lane 2, 1.0 mg Cr l^{-1}; Lane 3 , 3.5 mg Cr l^{-1}; Lane 4, 6.0 mg Cr l^{-1} .
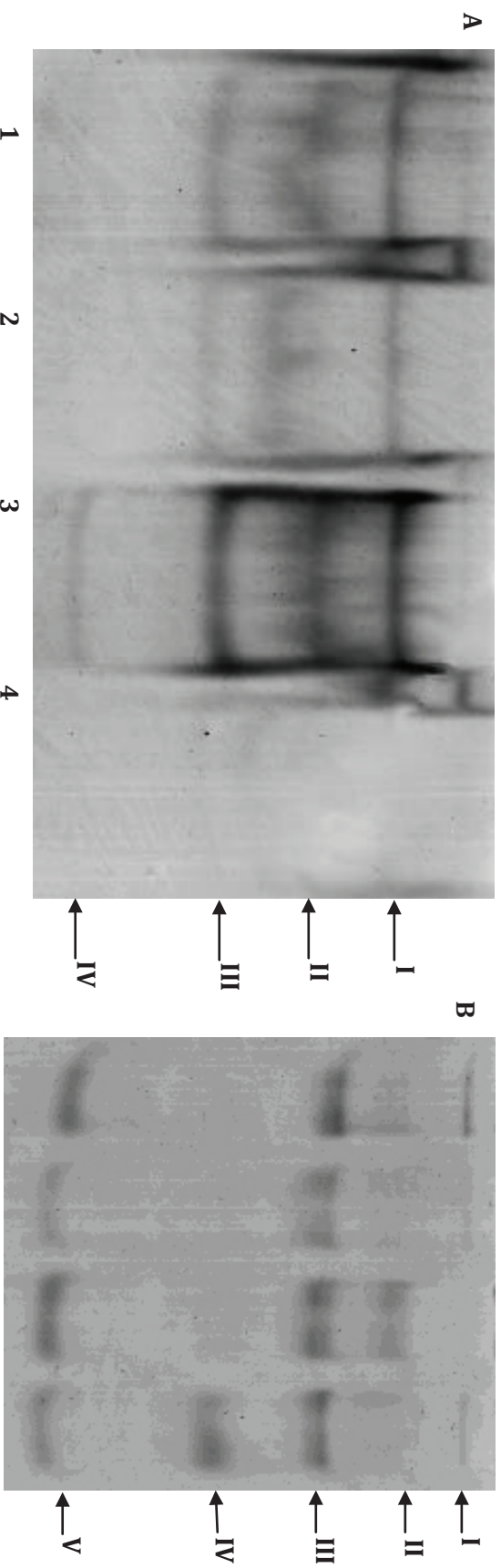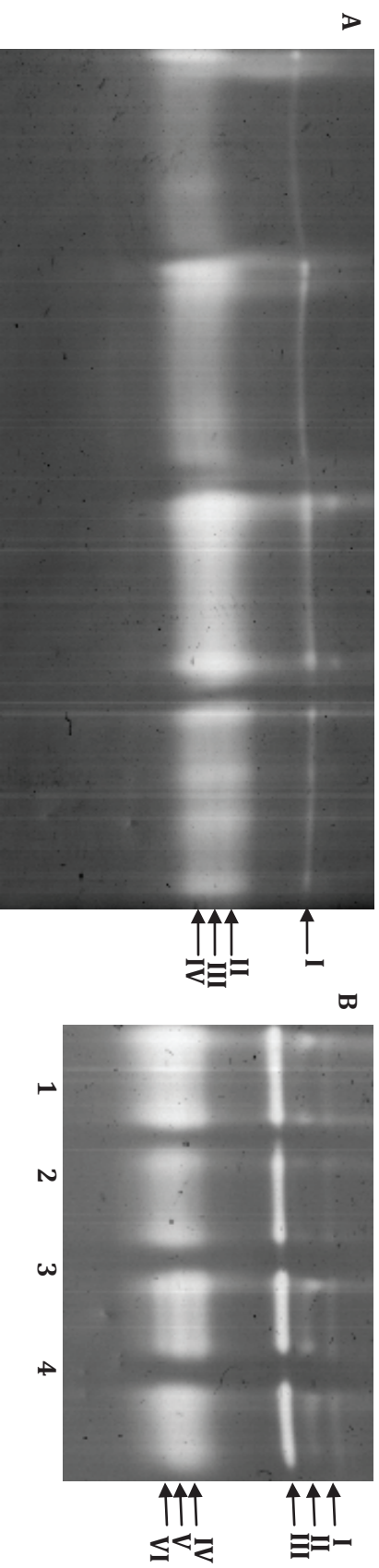

Figure 11. Activity staining for SOD (superoxide dismutase) isolated from roots (A) and leaves (B) of *P. stratiotes* after 36h of exposition to chromium. Lane 1, control; Lane 2, 1.0 mg Cr l^{-1}; Lane 3 , 3.5 mg Cr l^{-1}; Lane 4, 6.0 mg Cr l^{-1} .

The knowledge of how plants cope with metal-induced oxidative stress is of considerable importance in understanding the metal tolerance mechanisms evolved by plants (MONFERRÁN et al., 2009). The observed relation between Cr induced oxidative stress and capacity in *P. stratiotes* suggested the tolerance capacity of the plants to the metal depends on the balance of the factors favoring oxidative stress and factors reducing oxidative stress. However, like the results by us observed, the higher levels of enzymatic and non-enzymatic antioxidants suggested the reason for tolerating higher levels of metals (SINHA et al., 2005).

Tewari et al. (2008) suggested that *P. stratiotes* presented potential to be used as a phytoremediator species in the treatment of municipal sludge so as to ensure safety of populations residing near waste dump sites as well as the antioxidative enzymes can be used as heavy metal induced biomarker for assessing environmental damages.

The results presented in this study reflected system protective antioxidant located in various cell compartments to scavenging the ROS. Similar to our results, Sinha et al. (2009) suggested a pattern of variation and difference in responses of root and shoot tissues of the plant in terms of dominance of the biochemical variables at all concentrations and durations.

Our results also are in agreement with the literature that most of the chromium accumulates in the roots. Poor translocation of chromium to the leaves could be due to sequesterization of most of the chromium in the vacuoles of the root cells to render in non-toxic which may be a natural toxicity response of the plant (GANESH et al., 2008). Sinha et al. (2009) reported that a metal concentration gradient, thus build up between the root and shoots tissue of the exposed plants may lead to difference in setting up and magnitude of various responses in these tissues. Thus, Sinha et al. (2005) reported that the relation between Cr induced oxidative stress and capacity antioxidant in *P. stratiotes* suggest that the tolerance

capacity of the plants to the metal depends on the balance of the factors favouring oxidative stress and factors reducing oxidative stress.

Pistia stratiotes is used for phytoremediation of wastewater or natural water bodies polluted with heavy metals. The species exhibit different patterns of response to various heavy metals such as Ag, Cd, Cu, Hg, Ni, Pb and Zn, including chromium. Distinct level of growth inhibition and biomass production in this species can be observed upon 5 mM concentration of each of these metals, with almost all the elements being accumulated at high concentrations in the root system (SHAH; NONGYKINRH, 2007).

Similarly to the use of multivariate methods which have proven to be efficient for analyzing datasets with complex inter-relationships among the variables (SINHA et al., 2009) the use of the Central Composite Design (CCD) is advantageous; it can generate a maximum amount of information on the direct effect of test variables and their interactions while testing a minimum number of combinations (DE SCHAMPHELAERE et al. 2003). Statistical advice in current ecotoxicity test guidelines is in need of improvement, and the recent use of the factorial design in this area has presented promising results, which generate predictive models of toxicity to aquatic organisms. In this work, the developed models permitted the simulation of data, which together with other investigations of effect of chromium in *P. stratiotes* could be used to determinate potential adverse effects and applied to environmental risk assessment through the use of this species as a bioindicator and biomonitor of the environmental quality.

Based on the current study, it is possible to conclude that *P. stratiotes* promoted chromium differential bioaccumulation in roots in relation to leaves and therefore, the response for lipids peroxidation and for induced oxidative stress was more intense in the roots rather than leaves. This assessment was possible using the central composite design (CCD) and the response surface methodology (RSM) which provided information on the differential response standards, through the generation of mathematical models. The *P. stratiotes* plants are capable of tolerating high levels of chromium (VI) due to the fact that the damages caused by ROS in a given extension can be lightened through its antioxidative defense system. The answers from some of these enzymes were relatively fast, thus, they could be used as biomarkers. Hence, *P. stratiotes* could be indicated as a bioindicator, and also as a good biomonitor for chromium toxicity in aquatic ecosystems. Summarizing, it can be said that *Pistia stratiotes* is cosmopolite plant that presents rapid growth and high chromium accumulation, making its use desirable for the potential bioremediation of polluted environments.

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CAPÍTULO 8. EXPERIMENTAL DESIGN IN THE ASSESSMENT OF COPPER-INDUCED OXIDATIVE STRESS IN *PISTIA STRATIOTES* L.

ABSTRACT

The present investigation employed a Central Composite Design (CCD) and Response Surface Methodology (RSM) to evaluate the effect of different copper concentrations (2.0, 3.2, 6.0, 8.8 and 10.0 µg l^{-1}) applied by 12, 19, 36, 53 and 60h, on the bioaccumulation, chlorophyll content, and the induction of oxidative stress in *P. stratiotes* L. Statistical models were developed to describe these results, where the bioaccumulation in leaves was time-dependent. The decrease in the contents of chlorophyll was observed only in the most elevated concentrations of copper and exposure time, and the Chl *a* content was more affected than Chl *b*. There was variation in the response of the lipid peroxidation, as well as activity of catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11) and glutathione reductase (GR; EC 1.6.4.2). Thus, the oxidative stress was induced by copper in *P. stratiotes* showing toleration capacity to the imposed stress. The electrophoretic analysis of GR activity revealed five isoenzymes in roots and eight in leaves. Four isoenzymes were revealed on the SOD activity eletrophoretic analysis in roots and leaves, with expression differential of these isoenzymes in the different treatments. The activity of enzymes could be used as biomarker for environmental polluted, and the results demonstrated that *P. stratiotes* can be used as a phytoremediator, reinforcing its effectiveness in toxic metal remediation.

Key-words: bioaccumulation, chlorophyll, enzyms, experimental design, lipid peroxidation, metal, water lettuce.

RESUMO

O desenho experimental na avaliação do estresse oxidativo induzido por cobre em Pistia stratiotes L.

A presente investigação empregou o Delineamento Composto Central (DCC) e a Metodologia de Superfície de Resposta (MSR) para avaliar os efeitos de diferentes concentrações de cobre (2,0; 3,2; 6,0; 8,8 e 10,0 µg l^{-1}) aplicados por 12, 19, 36, 53 e 60h, na bioacumulação, conteúdo de clorofila e na indução de estresse oxidativo em *P. stratiotes* L. Modelos estatísticos foram desenvolvidos para descrever estes resultados, onde a bioacumulação de cobre nas folhas foi dependente do tempo. O decréscimo no conteúdo de clorofila foi observado apenas nas concentrações mais elevadas do metal e nos tempos de exposição mais prolongados, e o conteúdo de clorofila *a* foi mais afetado do que o de clorofila *b*. Houve uma variação na resposta da peroxidação de lipídios, assim como na atividade da catalase (CAT; EC 1.11.1.6), ascorbato peroxidase (APX; EC 1.11.1.11) e glutationa redutase (GR; EC 1.6.4.2). Sendo assim, o estresse oxidativo foi induzido pelo cobre em *P. stratiotes* demonstrando capacidade de tolerância ao estresse imposto. A análise eletroforética da atividade da GR revelou cinco isoenzimas em raízes e oito isoenzimas em folhas. Quatro isoenzimas foram reveladas na análise eletroforética da atividade de SOD em raízes e folhas, com expressão diferencial destas isoenzimas nos diferentes tratamentos. A atividade das enzimas poderia ser usada como biomarcadores de ambientes poluídos e os resultados demonstram que *P. stratiotes* pode ser usada como fitorremediadora, reforçando sua efetividade na remediação do metal tóxico.

Palavras-chave: alface d'água, bioacumulação, clorofila, desenho experimental, enzimas, metal, peroxidação de lipídios.

The pollution of aquatic ecosystems by exposure to toxic trace metals has assumed serious proportions and, because of this, is one of the main critical issues on environmental and public health (SRIVASTAVA et al., 2006; KANOUN-BOULÉ et al., 2009). Copper (Cu) is one of the most commonly used metals and its release into the environment arise from various anthropogenic activities including use of pesticides, fungicides and industrial wastes (MA et al., 2003; ANDRADE et al., 2004; YRUELA, 2005). This metal is known to be an essential micronutrient for plants growth being components of several proteins and enzymes involved in a variety of metabolic pathways (ELISABETTA; GIOACCHINO, 2004), but it can be a toxic element and is toxicity can be observed even at tissue contents slightly higher than is optimal levels (FERNANDES; HENRIQUES, 1991; OUZOUNIDOU et al., 1991). Bioconcentrations of Cu by aquatic macrophytes is of special concern to human welfare and for environmental protection and conservation (ORNES; SAJWAN, 1993). Toxicity of copper is mainly due to the existence of two readily interconvertible oxidation states (Cu^{2+} and Cu^+) making it highly reactive, and it can catalyze the formation of reactive oxygen species (ROS) through the Haber–Weiss reaction (TEISSEIRI; GUY, 2000; KANOUN-BOULÉ et al., 2009), which damage cell membranes, nucleic acids and chloroplast pigments (CHAOUI et al., 1997; FANG; KAO, 2000; TEWARI et al., 2002), and initiate peroxidation of membrane lipids (DAT et al., 2000). The protection to these ROS via antioxidant is complex and highly organized (SRIVASTAVA et al., 2006), and include several antioxidant enzymes such as catalase (CAT), peroxidases (POD), superoxide dismutase (SOD), glutathione reductase (GR), ascorbate peroxidase (APX) and non-enzymic scavengers, e.g. glutathione, carotenoids and ascorbat (NOCTOR; FOYER, 1998; TEISSEIRI; GUY, 2000; SRIVASTAVA et al.,

2004). Those enzymes of the detoxification machinery can serve as important markers of environmental pollution (FILHO et al., 2001) and a good correlation with pollutant levels further strengthens their utility as biomarkers (FERNANDES et al., 2002).

Pistia stratiotes is a free floating plant which accumulates a wide range of heavy metals, has a widespread habitat and potential to grow in nutrient rich environment having muddy water with low light intensities (ODJEGBA; FASIDI, 2004; SINHA et al., 2005). This macrophytes showed potential to be used as a phytoremediator as well as the antioxidative enzymes can be used as heavy metal induced biomarker for assessing environmental damages (TEWARI et al., 2008).

The use of the Central Composite Design (CCD) and Response Surface Methodology (RSM) analysis has been reported as an effective way of summarizing results from factorial experiments where all factors are quantitative, and it has been actually used in aquatic toxicology (EDGINTON et al., 2004). The use of CCD and MSR in aquatic environmental toxicology represents a powerful technique for investigating multivariate systems (because in the environment many factors may interact simultaneously). It reduces the number of experiments and repetitions without loss of statistical confidence (since it was possible to calculate the experimental error).

Considering previous investigations background, the aim of this work was to study the physiological responses in *Pistia stratiotes* to copper exposure by the Central Composite Design (CCD) and Response Surface Methodology (RSM). The copper toxicity was assessed through the analyses of antioxidant enzymes activities, lipid peroxidation, concentration of photosynthetic pigments and metals by the experimental design.

Plant material and treatment conditions

Plants of *Pistia stratiotes* L. were obtained from São Paulo littoral and maintained at the Laboratory of Ecotoxicology and Ecophysiology of Aquatic Organisms of the Water Resources and Applied Ecology Center (CRHEA) of the University of São Paulo (São Paulo State, Brazil). The macrophytes were cultivated in large containers (1000 L) with tap water and fertilizer KNP (4:14:8). The cultivation solution was maintained at pH 7±0.5 by titration with diluted HCl or NaOH.

Plants with similar size and growth stage were selected, washed thoroughly in a running tap water and acclimated for two days in laboratory in nutrient solution containing (mM): 1.25 $Ca(NO_3)_2.4H_2O$, 1.5 KNO_3, 0.5, KH_2PO_4, 0.5 $MgSO_4.7H_2O$, 0.05 H_3BO_3, 0.01 $MnCl_2.4H_2O$, 0.30 $CuSO_4.5H_2O$, 0.20 Na_2MoO_{24} and 0.5 NaFeEDTA(10%Fe) (ODJEGBA; FASIDI, 2004).

Tests were performed with one plant in 1 liter nutrient solution submitted to different nominal concentrations of copper (2.0-10.0 μg Cu l^{-1}) supplied as standard copper (Merk), and exposure time (12, 19, 36, 53 and 60 h) (Table 1). Plants in nutrient solution without copper served as a control. Both the control and the treated solutions were maintained at pH 5.5 by titration with diluted HCl or NaOH, this low pH helped keep metal in solution and available for absorption by plant roots (ODJEGBA; FASIDI, 2004). The macrophytes during the test were maintained at 26±2°C and under a constant irradiance of 7.000 lux provided by cool-white fluorescent lamp.

Copper quantification

Harvested leaves and roots *P. stratiotes* were washed thoroughly with mili-Q water, blotted and oven dried for 48 h at 60 ºC. The preparation of samples for copper estimation was carried out by acid digestion (HNO_3 and H_2O_2) at 120 °C and then diluted with mili-Q water, and all analytical procedures were accompanied by analytical blank (APHA, 1995). The measured concentration of metal was, expressed as µg Cu mg^{-1} dry weight. All the samples were analyzed in triplicates by graphite-furnace atomic absorption spectrometry (Varian AA 220). The recoveries of metals from the plant tissues were found to be more than 94%, the detection limit was 0.67 µg l^{-1} and the quantification limit was 2.23 µg l^{-1}.

Lipid peroxidation

The level of lipid peroxidation in plant roots and leaves was determined by estimation of the malondialdehyde (MDA) content based on the method of Heath and Packer (1968). Thiobarbituric acid-reacting (TBARS) substances representing the lipid peroxidation product were extracted by homogenization of leaves and roots from *P. stratiotes* (300 mg) in a pestle and mortar with 20% (w/v) insoluble polyvinylpyrrolidone (PVPP) and 1.3 ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10 000g for 5 min., and 1 ml 0.5% TBA in 25% TCA at 250 µl of the supernatant was added. The mixture was heated at 95 °C for 30 min and the reaction was stopped by quickly transferring the mixture to an ice bath. The absorbance of the TBARS was determined spectrophotometrically at 535 nm. Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm, and the concentration of TBARS was calculated using the absorbance coefficient 1.55 x 10^{-5} mol^{-1} cm^{-1}.

Assay of antioxidant enzymes

All biochemical analyses were performed at 4 °C unless stated otherwise. The samples of leaves and roots from *P. stratiotes* were homogenized (300 mg) in a mortar with a pestle with 100 mM chilled potassium phosphate buffer (pH 7.5) containing 1mM ethylenediaminetetraacetic acid (EDTA), 3 mM $_{DL}$-dithiothreitol and 5 % (w/v) insoluble PVPP. Homogenate was centrifuged at 10 000g for 30 min and the supernatant was stored in aliquots at -80 °C and was used to measure the activities of CAT, APX and GR. Proteins content was measured according to Bradford (1976) using serum albumin as the standard protein.

The total activity of CAT (EC 1.11.1.6) in leaves and the roots from *P. stratiotes* was determined spectrophotometrically by monitoring the degradation of H_2O_2 at 240 nm for1 min against a plant extract-free blank (AZEVEDO et al., 1998). APX (EC 1.11.1.11) total activity was measured in leaves and the roots by the method of Nakano and Asada (1981), by monitoring the rate of ascorbate oxidation at 290 nm at 30 °C. The activity was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1}. Total GR (1.6.4.2) activity leaves and the roots were determined spectrophotometrically as described by Azevedo et al. (1998). The reduction of GSSG (Oxidized Glutathione) was followed by monitoring the increase in absorbance at 412 nm for 2 min.

Polyacrylamide gel electrophoresis (PAGE)

SOD and GR were evaluated by electrophoretic analysis under non-denaturing condition in 12 % polyacylamide gels as described by Medici et al. (2004).

GR activity in native PAGE gels was determined as described by Lee and Lee (2000) with modifications as described by Medici et al. (2004). SOD activity was determined as described by Beauchamp and Fridovich (1971) and modified by Azevedo et al. (1998).

Photosynthetic pigments estimation

Leaves discs of *P. stratiotes* were cut with ca 200 mm^2 surface. These discs were extracted with 2 ml aqueous acetone 80% in a mortar with pestle. The homogenate, combined with a further three washings of the pestle and mortar (each of 1.5 ml) with the same solvent, was centrifuged at 2500 rpm for 10 min. The pellet was then extracted with a further 1 ml of solvent and the pooled supernatants adjusted to a final volume of 8 ml (PORRA et al., 1989). The absorbance at the major absorption peak of chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) and Chlorophyll total (Chl *a* + Chl *b*) were measured in Spectrophotometer and the concentrations in µg l^{-1} were then calculated according to Porra et al. (1989).

Statistical analysis

Toxicity tests with *P. stratiotes* were conducted in 11 experiments for the study of two parameters (see matrix in table 1). The model studied is a 2^2 experimental design. We selected time of exposure (X1) and chromium concentration (X2).

Runs	Coded setting levels		Actual levels	
	x1= time; x2= [Cu]		X1= time (h); X2= [Cu] (µg l⁻¹)	
	x1	x2	X1	X2
1	-1	-1	19	3.2
2	-1	1	19	8.8
3	1	- 1	53	3.2
4	1	1	53	8.8
5	0	0	36	6.0
6	0	0	36	6.0
7	0	0	36	6.0
8	0	- 1.41	36	2.0
9	0	1.41	36	10.0
10	-1.41	0	12	6.0
11	1.41	0	60	6.0

The experimental results of the response surface methodology were fitted with a second-order polynomial equation (4) by a multiple regression technique.

$$Y = b_0 + b_1 \, x_1 + b_2 \, x_2 + b_{11} \, x_1^{\,2} + b_{22} \, x_2^{\,2} + b_{12} \, x_1 \, x_2 + \varepsilon \qquad (4)$$

Y is the predicted response, b_0, b_1, b_2, b_{11}, b_{22}, b_{12} are constant coefficients, and x1, x2 are the coded independent variables or factors.

The test factors were coded according to the following regression equation (5):

$$x_i = \left(\frac{X_i - X_0}{\Delta X_i} \right) \qquad (5)$$

where x_i is the coded value and X_i is the actual value of the ith independent variable, X_0 is the actual value at the center point, and ΔXi is the step change value. Statistic 7.0 software (Statsoft, USA) was used for regression and graphical analysis of the data.

The quality of fit of the second-order model equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by an *F*-test. The significance of the regression coefficients was tested by a *t*-test.

8.3. RESULTS AND DISCUSSION

Stress parameter and toxic element concentration

The results obtained from the 2^2 factorial central composite designs were used to assess the copper toxicity through the analyses of metals concentration in leaves of *P. stratiotes* and photosynthetic pigments content (chlorophyll *a* and *b*). The results obtained from experimental design are shown in Table 2. In general, there is a tendency to increase the accumulation of metals and it is observed that this increase is proportional to the increase in metal concentrations in solution and time-dependant (SRIVASTAVA et al., 2006; MONFERRÁN et al., 2009). This assertion confirms our results, where in the leaves the high accumulation was observed in more prolonged exposure time. The model obtained for copper concentration represents that it was reasonably significant to time quadratic ($p < 0.05$). Despite the differences were not significant for the copper accumulation in roots, but now in

the initial exposure times (12, 19 and 36h) elevated concentrations of metal were observed and the accumulation was bigger in roots than in the leaves.

Since the response surface analysis of the copper concentration in leaves presented the coefficient of determination of 82% ($p < 0.05$) it verified the adequacy of using the regression model for describing the contour surface (Table 3 and figure 1). A response surface analysis of the copper concentration in leaves presented the coefficient of determination of 82% ($p < 0.05$) verified the adequacy of using the regression model for describing the contour surface (Table 3 and figure 1), where the cove of curves explain these results.

Table 2. The mean experimental design responses with the results obtained from the activity of enzymes catalase (CAT), ascorbate peroxidase (APX) and Glutathione Reductase (GR) in $\mu mol\ min^{-1}\ mg\ protein^{-1}$, the estimative of lipid peroxidation through TBARS content in $M\ g^{-1}$, the copper concentration in $mg\ L^{-1}\ dry\ wt^{-1}$ in leaves, and the chlorophyll content in $\mu g\ L^{-1}\ f\ wt^{-1}$.

| Runs	Roots		Leaves					
	CAT	GR	CAT	GR	TBARS	[Cu]	Chl a	Chl b
1	20.55	3.87	204.38	2.42	1,03	2.05	239,73	669,79
2	48.88	2.19	136.22	3.35	0,50	2.81	359,47	767,82
3	88.18	6.72	17.21	1.03	0,76	3.27	283,32	587,74
4	31.32	2.79	0.00	0.56	0,86	3.31	256,72	704,54
5	302.33	7.69	26.07	1.36	1,16	1.70	289,74	586,44
6	344.44	7.58	17.37	1.26	1,05	1.83	286,18	542,30
7	323.38	7.64	21.72	1.15	1,30	1.96	287,96	564,37
8	44.10	2.33	493.17	4.54	1,12	5.21	220,28	550,44
9	63.32	3.37	92.68	0.88	1,07	1.61	365,41	770,42
10	18.03	1.85	159.47	1.53	0,96	3.80	273,00	774,26
11	114.42	2.67	85.64	0.87	0,94	2.54	300,81	696,83

Copper concentrarion in leaves
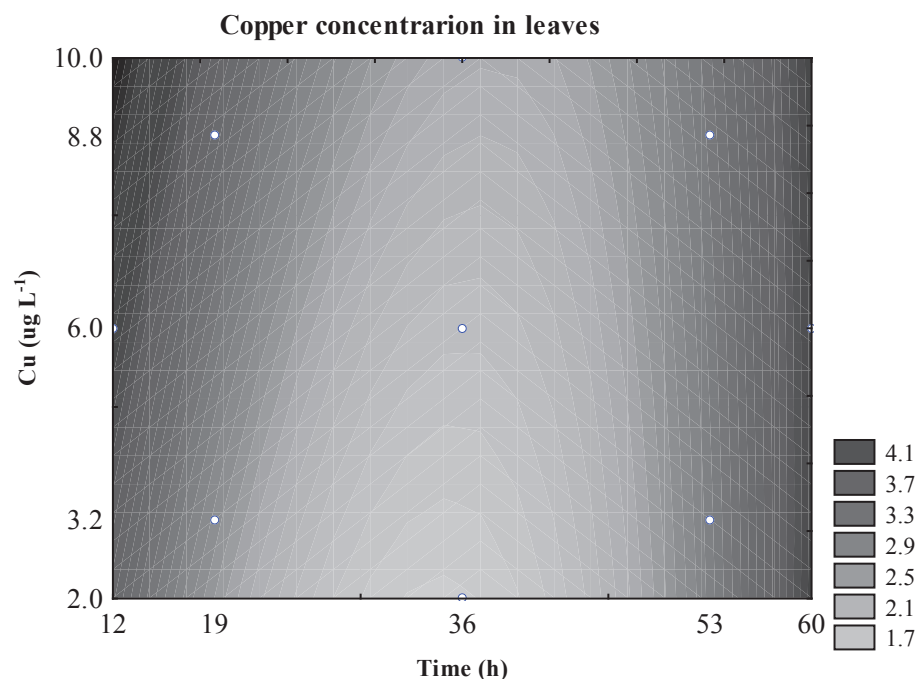
Figure 1. Contour surface plot of bioaccumulation in leaves concentration as a function of copper concentration (2.0-10.0 µg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

In agreement with our results, Upadhyay and Panda (2009) also observed copper accumulation in both roots and leaves of *P. stratiotes*, which showed a dose dependent increase, but differently than our results, this increase was higher in leaves as compared to roots. According to Upadhyay and Panda (2009) the higher accumulation of Cu in *Pistia* plants may result in the activation of a putative Cu transporter and also may result in the distortion of plasma membrane.

Significant accumulation of copper has been observed in other aquatic plants like *Ceratophyllum demersum* (DEVI; PRASAD, 1998), *Lemna trisulca* (PRASAD et al., 2001) and *Vallisneria spiralis* (VAJPAYEE et al., 2005).

The accumulation of metals in aquatic macrophytes often induces important metabolic disturbances and particularly chlorophyll degradation (PRASAD et al., 2001; LI et al., 2006;

PERALES-VELA et al., 2007). In spite of that, the chlorophyll *a* (Chl *a*) and *b* (Chl *b*) content was not significantly affected by copper concentration, in this study, the Chl *a* content was more affected than Chl *b* and both showed different behavior in the statistical analysis (Table 3). The response surface analysis of chlorophyll *a* and *b* content was performed indicating that circa 86 and 96% of the variability on the data could be explained by the regression model describing the contour surface, respectively (Figures 2 and 3). The model of Chl *a* content represents that it was significantly affected by linear exposure time, and linear copper concentration and quadratic just like by interaction between two factors (time and metal concentration). Anyway there was increase in chlorophyll content in relation to control plants in the initial exposure time, the reduction was observed with 53 and 60 h of exposure to more elevated Cu concentrations, which can be observed in the presence of curvature in the regions of interest in the contour surface (Figure 2).
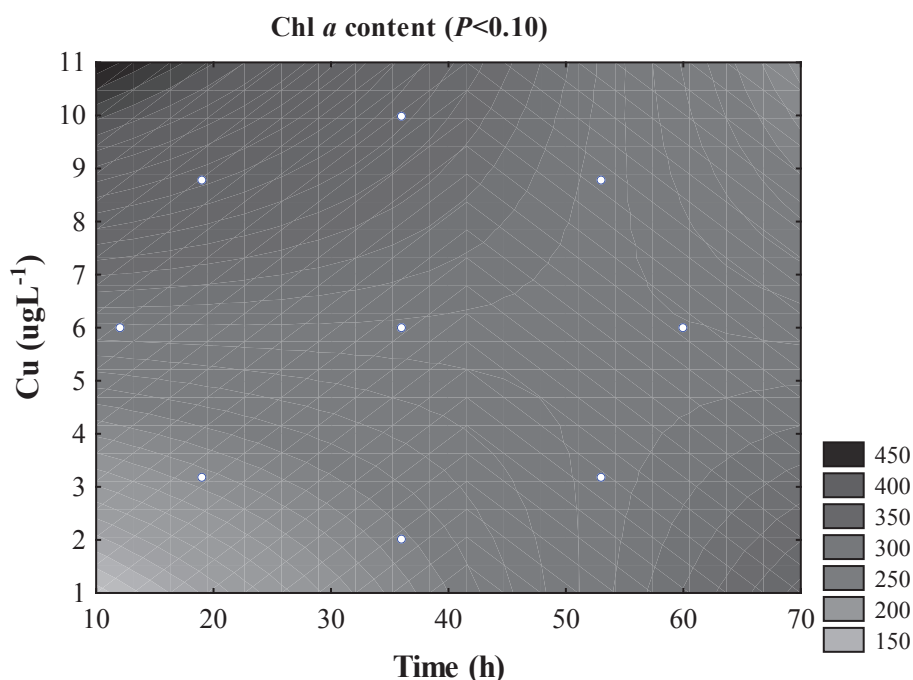

Figure 2. Contour surface plot of chlorophyll *a* content as a function of copper concentration (2.0-10.0 µg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

Table 3. Statistical analysis of different parameters analyzed by experimental design. The activity of enzymes catalase (CAT) and Glutathione Reductase (GR) are in µmol min⁻¹ mg protein⁻¹, the estimative of lipid peroxidation through TBARS content in M g⁻¹ f wt, the copper concentration [Cu] in µg L⁻¹ g dry wt⁻¹, and the chlorophyll content in µg L⁻¹ g f wt⁻¹.

| | Coefficient estimate (± Standard error) | | | | | | | | | |
| | Roots | | | Leaves | | | | | | |
Terms	CAT	GR	APX	CAT	GR	APX	TBARS	Chl a*	Chl b	[Cu]
b_0	*-1006.40 (±95.6)*	*7.630 (±0.8)*	*423.245 (±53.2)*	*904.401 (287.0)*	*1.256 (±0.07)*	*140.084 (±36.2)*	*1213.48 (±96.0)*	44.987 (±85.2)	*1.634 (±0.0)*	*1.829 (±0.4)*
b_1	*40.230 (±3.3)*	0.349 (±0.5)	21.470 (±32.7)	*-38.689 (±9.9)*	*-1.053 (±0.04)*	*-64.989 (±22.2)*	7.473 (±58.9)	4.910 (±2.9)	*-0.0268 (±0.0)*	-0.332 (±0.2)
b_2	*-0.520 (±0.04)*	*-2.442 (±0.6)*	*-177.773 (±39.0)*	*0.433 (±0.1)*	*0.864 (±0.05)*	3.566 (±26.5)	*-192.41 (±70.3)*	-0.006 (±0.0)	*0.00022 (±0.0)*	*1.236 (±0.3)*
b_{11}	*199.300 (±19.9)*	-0.826 (±0.5)	-12.475 (±32.7)	-24.425 (±59.7)	0.057 (±0.04)	12.469 (±22.2)	-62.599 (±58.9)	*39.120 (±17.7)*	*-0.1984 (±0.0)*	0.265 (±0.2)
b_{22}	*-15.100 (±1.4)*	*-1.975 (±0.6)*	*-160.526 (±39.0)*	0.881 (±4.2)	*-0.222 (±0.05)*	-14.297 (±26.5)	-117.89 (±70.3)	0.152 (±1.3)	*0.0116 (±0.0)*	0.013 (±0.3)
b_{12}	-0.450 (±0.3)	-0.561 (±0.7)	-37.401 (±46.1)	0.268 (±0.8)	*-0.348 (±0.06)*	-2.121 (±31.3)	156.839 (±83.2)	*-0.769 (±0.2)*	*0.0017 (±0.0)*	-0.181 (±0.3)
R^2	0.979	0.857	0.859	0.844	0.995	0.650	0.723	0.856	0.881	0.821
F-value	69.87	23.92	24.54	21.96	279.29	16.69	23.47	23.74	7.38	41.14
F stat table	4.53	4.46	4.46	4.46	4.53	5.12	5.12	4.54	5.05	5.12
F cal/Ftab	15.42	5.36	5.5	4.92	61.65	3.26	4.58	5.23	1.46	8.04

Values of terms in bold and italic are significantly different at $P < 0.05$, and * Chl a content values of terms in bold and italic are significantly different at $P < 0.10$.
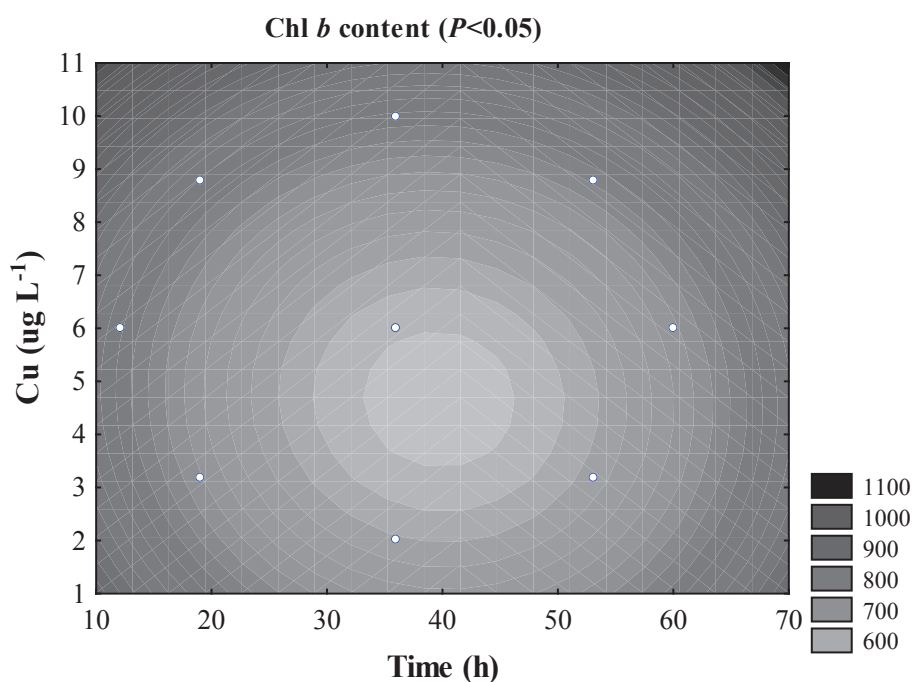
Figure 3. Contour surface plot of chlorophyll *b* content as a function of copper concentration (2.0-10.0 µg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*

The content of Chl *b* was affected by more prolonged exposure time and more elevated copper concentrations, according to model of regression describing by contour surface (Figure 3). The statistical analysis showed that all factors were significant ($p < 0.05$) (Table 3), which indicates that they act as limiting factors and even small variations in their values will alter the chlorophyll b content to a considerable extent. Unlike Chl *a*, Chl *b* content showed a reduction in the initial exposure time (12 to 36h) as the copper concentration increased, according to contour surface (Figure 3).

Upadhyay and Panda (2009) also working with *P. stratiotes* and copper (0 to 6.4 mg Cu l^{-1}) related that initially there was increase but then it decreased gradually with exposure time. Results similar to these have been reported in other aquatic plants (PANDA; CHOUDHURY, 2005; PANDA, 2007). Srivastava et al. (2006) related no significant effect on the level of

chlorophyll *a* and *b* was observed up to 0.06 mg Cu l^{-1} till 4 days, beyond which their contents declined with more severe effect on Chl *a* than Clh *b*. Hou et al., (2007) also related that the degradation rate of chlorophyll *b* in *Lemna minor* under heavy metal stress (Cu^{2+} and Cd^{2+}) was lower than that of chlorophyll a and its damage is greater. It is important to record that Chl *a* is one the most important center pigments in photosynthesis and therefore the decrease can inhibit photosynthesis greatly.

The level of lipid peroxidation in plants is obtained by estimation of the malondialdehyde (MDA) content through measures of TBARS, and MDA is the decomposition product of polysatured fatty acids of membranes. In general, its increase shows plants are under high-level antioxidant stress (HOU et al., 2007). The results obtained from design experimental to TBARS content in leaves of *P. stratiotes* are shown in Table 2. A response surface analysis of the TBARS in roots and leaves was performed, but the differences were significant only for the leaves. The TBARS content in roots were bigger in relation to control plants only in the higher copper concentrations and after 36h of exposure. The coefficient of determination to TBARS content in leaves was 72% ($p < 0.05$) which verified the adequacy of using the regression model for describing the contour surface (Figure 4). In this model, only time quadratic was significant in leaves (Table 3), which indicates that even small variations in their values will alter the TBARS content in leaves to a considerable extent. The curvatures in a wide region of contour surface (Figure 4) reveal the occurrence of an increasing punctual of TBARS content in relation to control plants since 12h of exposure and 2.0 μg Cu L^{-1} to 53h and 8.8 μg Cu L^{-1}, but in general there was reduction in this factor in relation to control plants.

TBARS content in leaves
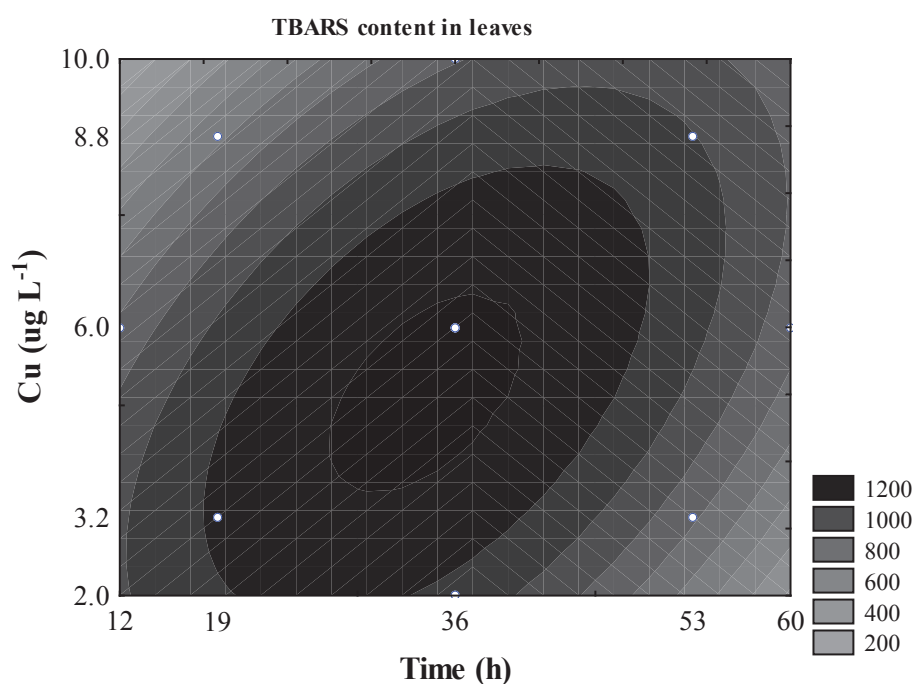
Figure 4. Contour surface plot of TBARS content as a function of copper concentration (2.0-10.0 μg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

Srivastava et al. (2006) related that the oxidative stress imposed to the plants of *Hydrilla verticillata* upon copper exposure was evident by significant in MDA content only at higher concentrations. Similarly, Sinha et al. (2009) also observed significant increase of MDA in higher concentrations by chromium stress imposed to plants of *Pistia stratiotes*. These results are in conformity with those observed for *Ceratophyllum demersum* (DEVI; PRASAD, 1998) and *P. stratiotes* (SINHA et al., 2005). On the other hand, they are in disagreement with the studies realized by Gupta et al. (1996) that demonstrate decrease in MDA content in copper stressed *H. verticillata* plants as compared to control plants.

Antioxidant enzymes activity

Redox cycling between Cu^{2+} and Cu^+ catalyzes production of hydroxyl radicals from superoxide and hydrogen peroxide by the Haber-Weiss reaction and thus enhances the production of ROS (TEISSEIRI; GUY, 2000; KANOUN-BOULÉ, 2009). Protection against enhanced ROS generation is achieved through stimulation of both enzymatic and molecular antioxidants (SRIVASTAVA et al., 2006). In this study, various antioxidant enzymes showed significant changes in their activity in function of exposure time to different copper concentrations in *P. stratiotes* (Table 2).

CAT scavenges the reactive oxygen species in plant cells and participates in the main defense system against accumulation and toxicity of hydrogen peroxide. A response surface analysis of the CAT activity in roots and leaves of *P. stratiotes* was performed. The elevated coefficient of regression (98 and 84%, in roots and leaves, respectively) verified the adequacy of using the regression model for describing the contour surface with 95% of confidence (Figures 5 and 6).

Figure 5. Contour surface plot of CAT in roots specific activity as a function of copper concentration (2.0-10.0 µg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

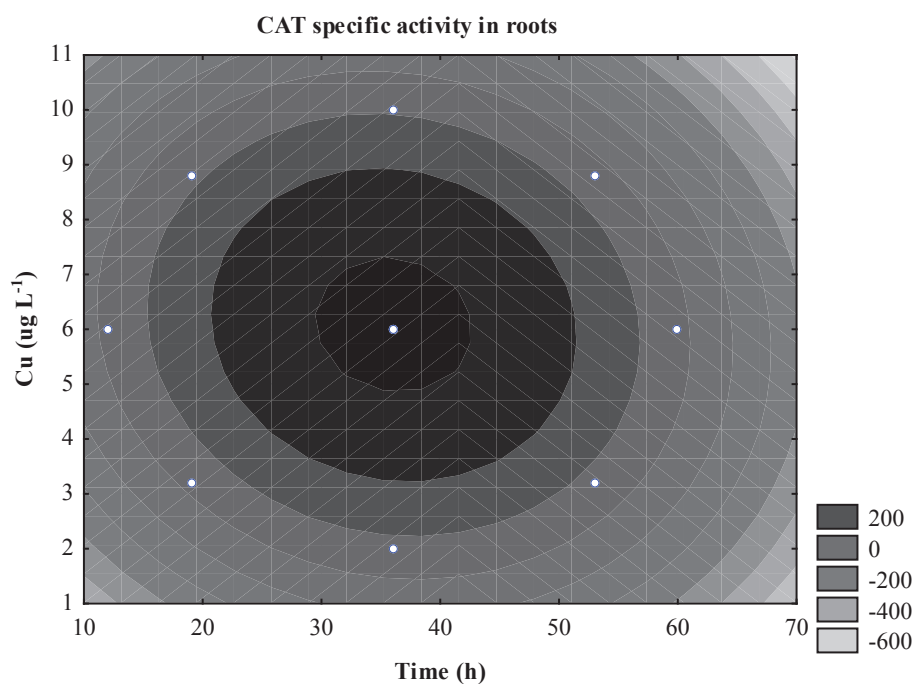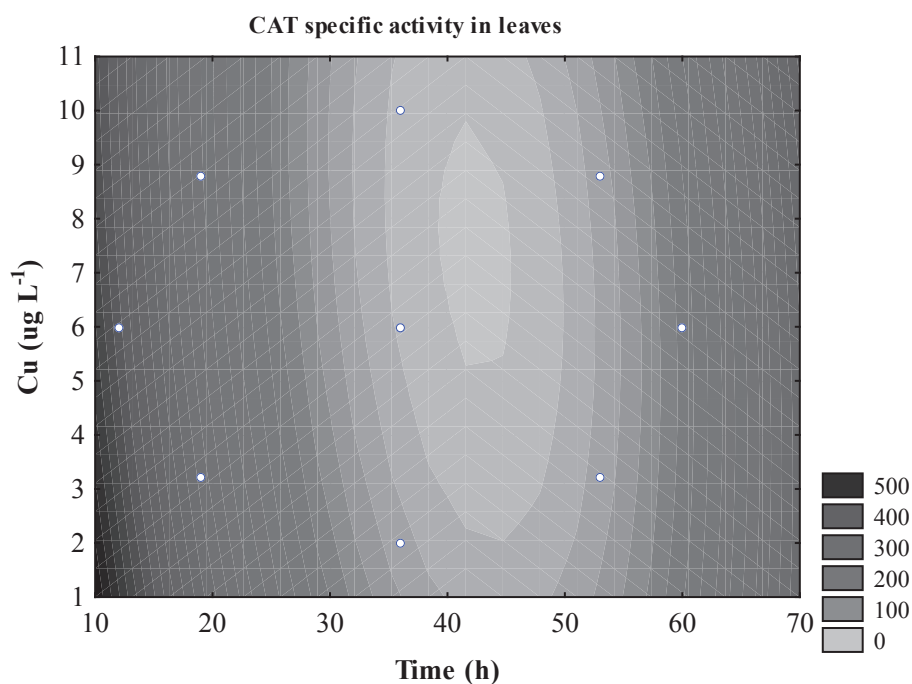**Figure 6.** Contour surface plot of CAT in leaves specific activity as a function of copper concentration (2.0-10.0 µg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

In roots all terms were significant, time and chromium linear and quadratic as well as the interaction between them (Table 3). This indicates that when they are significant the quadratic terms, they act as limiting factors and even small variations in their values will alter the enzyme´s activity to a considerable extent. The contour surface in roots (Figure 5) indicates the region of higher activity of CAT in the central point (36h and 6.0 µg Cu L^{-1}), where the values were around 500% more elevated that control plants. In leaves, only time linear and quadratic were significantly different (Table 3). The CAT activity in leaves of *P. stratiotes* were greater as compared to control plants in the smaller time of exposure (12, 19 and 36h) and the higher time (60h) in practically all concentrations of chromium, as can be observed in the contour surface (Figure 6). Similarly, Upadhyay and Panda (2009) related that CAT´s activity showed initial increase after 12h of Cu exposure but gradually decreased after 18h. Srivastava et al. (2006) also observed similar results working with *Hydrilla verticillata* upon copper stress.

On the other hand, Ganesh et al. (2008) reported that the CAT activity gradually decreases with the increase of chromium concentrations in *P. stratiotes*. Kanoun-Boulé et al. (2009) working with *Lemna minor* upon copper stress observed that relative CAT activity was lower with increasing concentrations of copper. According to Cakmak (2000) there is a sensitivity of enzyme to the $O_2^{-\bullet}$ a radical produced under Cu stress as it is known the enzyme activity can be inhibited by increased levels these radicals. Other possibility that could explain the loss in CAT activity might also ascribe to the degradation caused by induced peroxisomal proteases (SANDALIO et al., 2001).

GR is another important enzyme constituent of the antioxidative defense system that helps in maintaining a high GSH/GSSG ratio, crucial for protection against oxidative damage (MISHRA et al., 2006). The results obtained from design experimental to GR activity in *P.*

stratiotes are also shown in Table 2, and the analysis of enzyme´s activity in roots and leaves demonstrated distinct behavior. The response surface analysis to GR activity was performed, indicating that 86% and 99% of the variability on the data could be explained by the regression model describing the contour surface (Table 3 and figures 7 and 8). The statistical analysis was significantly affected by time and copper quadratic in roots ($p < 0.05$), i.e., these terms influence the activity of enzyme in a relevant way (Table 3). The GR activity in roots was more intense mainly in the center point (36h and 6.0 µg Cu l^{-1}) where the results were bigger in relation to control plants, which can be observed in the presence of curvature in the regions of interest in the contour surface (Figure 7). The GR activity decrease as compared to control plants at 19h (8.8 µg Cu l^{-1}) and at 60h (10.0 µg Cu l^{-1}). In the leaves, the statistical analysis was not significant only to chromium concentration linear. The terms of model: time of exposure (linear and quadratic), chromium concentration (quadratic) and the interaction between them are significant; therefore it means that these factors significantly affect the activity of enzyme. The activity of GR was more elevated than control plants in the initial exposure time and the higher concentrations per region was darker in performed contour surface (Figure 8).

It has been confirmed in many studies that an excess in copper can promote and stimulate generation of Fenton-type reactive oxygen species, leading to an increase in the activity of antioxidant enzymes (DEVI; PRASAD, 1998). Higher GR activity in leaves could maintain a higher GSH/GSSG ratio in leaves than roots, which resulted in more oxidative damage to roots at higher concentrations (MISHRA et al., 2006).

GR specific activity in roots
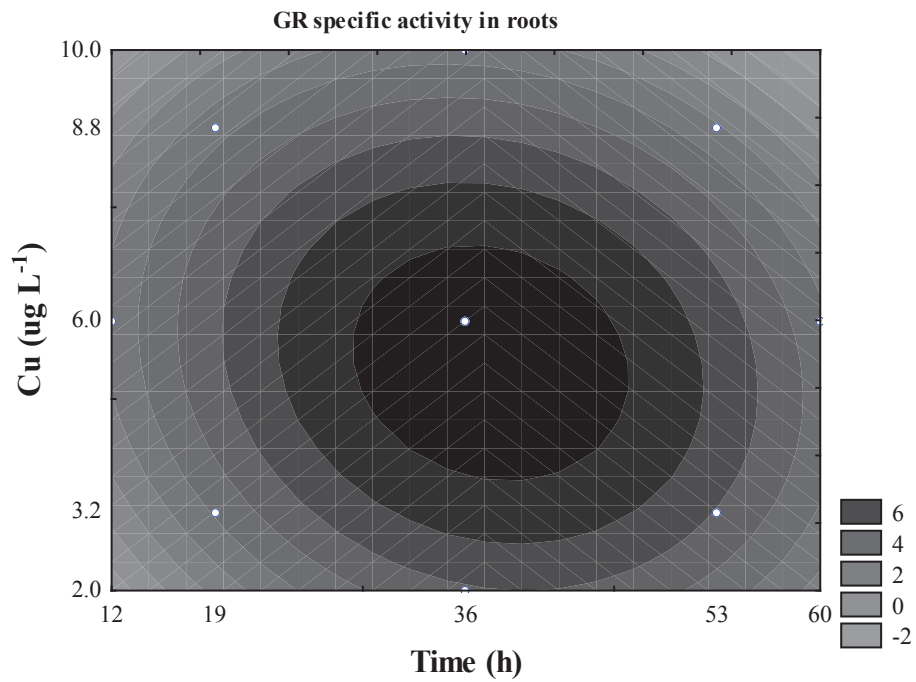
Figure 7. Contour surface plot of GR in roots specific activity as a function of copper concentration (2.0-10.0 µg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.
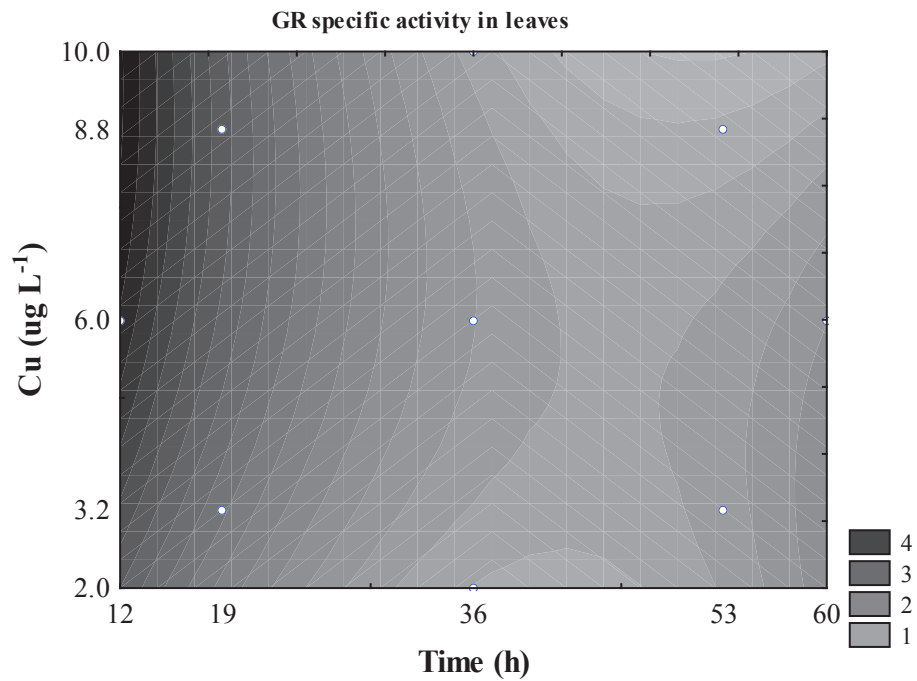

GR specific activity in leaves

Figure 8. Contour surface plot of GR in leaves specific activity as a function of copper concentration (2.0-10.0 µg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

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Monferrán et al. (2009) and Srivastava et al. (2006) observed increased activities of GR at variable copper concentrations, depending on the exposure time, followed by inhibition at higher concentrations or times. Probably because GR is extremely sensitive to inhibition by heavy metal ions like Cd^{2+}, Cu^{2+}, Fe^{3+} and by compounds that react with the –SH groups due to the presence of thiol groups at the active site of the enzyme (NAGALAKSHMI; PRASAD, 2001).

It has been shown that the number of GR isoenzymes varies among plant species (GRATÃO et al., 2005). The activity of GR in roots of *P. stratiotes* by electrophoretic analysis staining revealed at least five isoenzymes (bands I-V) (Figure 9A). Bands I to III exhibit expression in the control plants and at higher concentration of copper (lane 1 and 4). In lane 2, band III exhibit weak activity of GR. Bands IV and V showed low intensity expression of isoenzymes in all treatments with copper including control plants except at lane 3. In leaves of *P. stratiotes* GR activity staining revealed eight isoenzymes (bands I-VIII) (Figure 9B). Most of GR´s isoenzymes, exhibited intense activity (higly electropositive) as compared to control plants (lane 1), in this case, moderate activity of bands 5, 7 and 9 can be observed. The band I did not exhibit activity at 6.0 µg Cu l^{-1} (lane 3), and in this treatment band V exhibited a less intense expression.

Figure 9. Activity staining for GR isolated from roots (A) and leaves (B) of *P. stratiotes* after 36h of exposition to copper. Lane 1, control; Lane 2, 2.0 µg Cu l[-1]; Lane 3 , 6.0 µg Cr l[-1]; Lane 4, 10.0 µg Cr l[-1].
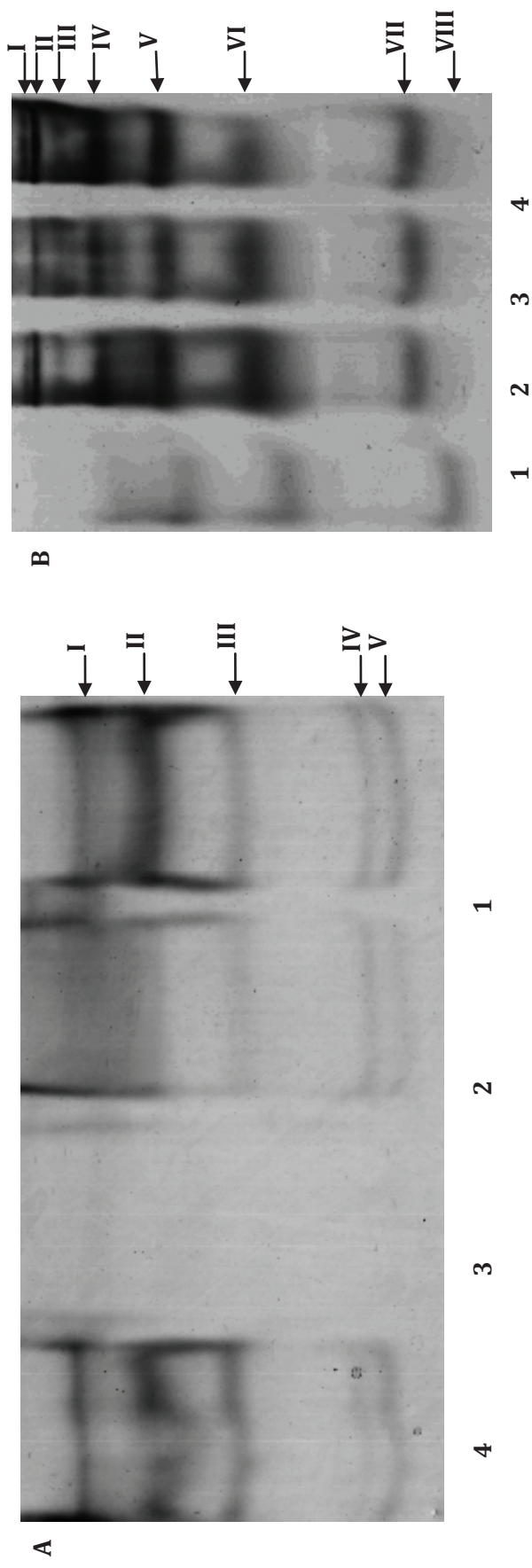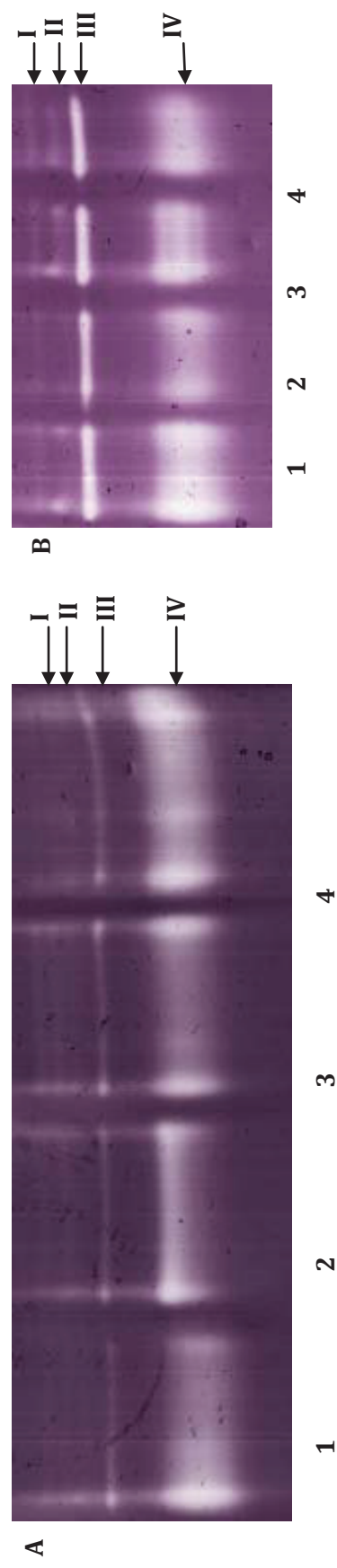

Figure 10. Activity staining for SOD isolated from roots (A) and leaves (B) of *P. stratiotes* after 36h of exposition to copper. Lane 1, control; Lane 2, 2.0 µg Cr l[-1]; Lane 3 , 6.0 µg Cr l[-1]; Lane 4, 10.0 µg Cr l[-1].

Among enzymatic scavengers, SOD is involved in the detoxification of $O_2^{-\bullet}$ radicals (UPADHYAY; PANDA, 2009). In this work the activity of SOD will be presented only by electrophoretic analysis. SOD activity in roots of *P. stratiotes* staining revealed at least four isoenzymes (bands I-IV) (Figure 10A). Bands I and II exhibit low intensity expression in all treatments including the control plants, likewise for bands III and IV, but these exhibit intense activity of SOD isoenzymes. In leaves of *P. stratiotes*, SOD activity staining also revealed four isoenzymes (bands I-IV) (Figure 10B), with expression differential these isoenzymes in the different treatments.

In the point center of design experimental (at 36h and 6.0 µg Cu l^{-1}) the isoenzymes presented weak expression as compared with other treatments. Band II also exhibited weak expression in the control plants (lane 1) and treatment at 10.0 and 6.0 µg Cu l^{-1} (lane 4). Bands III and IV exhibited expression more intense (more electropositive) in leaves of *P. stratiotes* including in the control plants.

Upadhyay and Panda (2009) related that the SOD activity showed minor increase after 18h in both roots and leaves of *P. stratiotes* upon copper stress, while after 24 h of treatment, activity was increased maximum in leaves and roots at 6.4 mg Cu l^{-1} as compared to control. According to these authors, the induction of SOD by copper may indicate an acclimation response of the plants, and its increase is related to oxidative stress tolerance.

As said before, SOD is responsible for the dismutation of $O_2^{-\bullet}$ to form H_2O_2 and O_2, whereas CAT, APX, GOPX (guaiacol peroxidase) are enzymes that catalyze the conversion of H_2O_2 to water and O_2. APX has been shown to play an important role in the detoxification of H_2O_2 under abiotic stress (GRATÃO et al., 2005). The results obtained from design experimental to APX activity in roots and leaves of *P. stratiotes* are shown in Table 2. The

response surface analysis of the activity of APX in roots and leaves was performed, and it showed distinct behavior in the statistical analysis (Table 3). The coefficient of regression (86 and 65%, in roots and leaves, respectively) verified the adequacy of using the regression model for describing the contour surface with 95% of confidence (Figure 11 and 12). The APX activity in roots showed higher values in the region of center point (36h and 6.0 µg Cu l^{-1}), this can be observed in the circulars curves presented in the contour surface graphic (Figure 11). The time of exposure quadratic was the significant term ($p < 0.05$), i.e. the APX activity will be strongly influenced by this parameter.
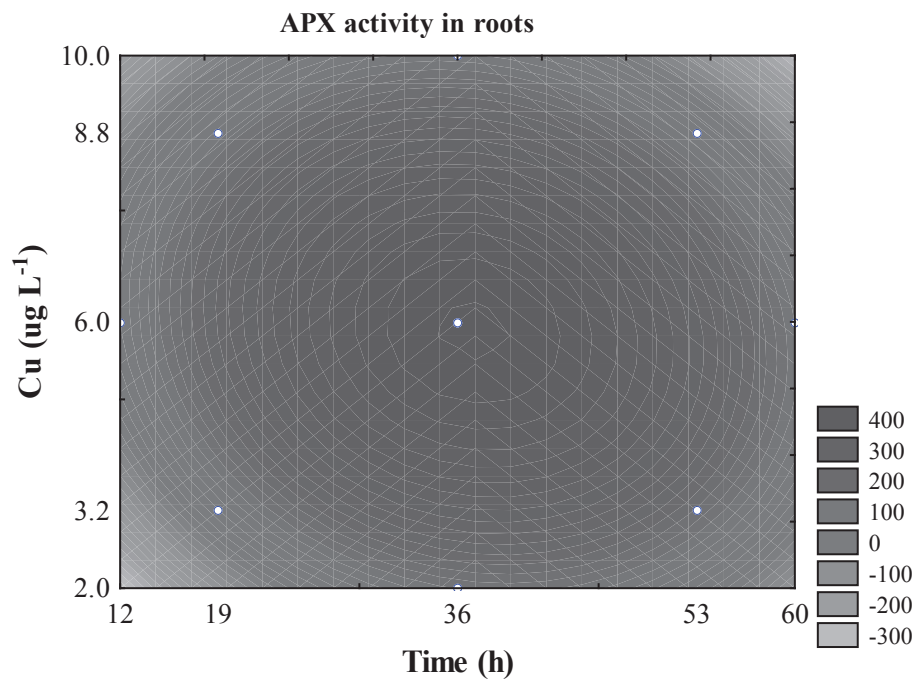
Figure 11. Contour surface plot of APX specific activity in roots as a function of copper concentration (2.0-10.0 µg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

APX activity in leaves
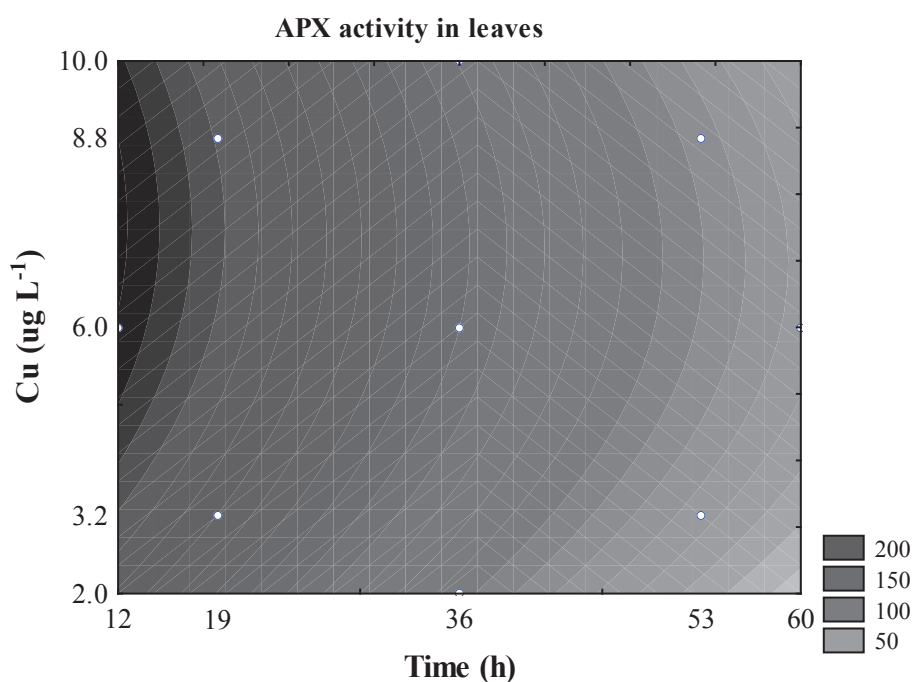
Figure 12. Contour surface plot of APX specific activity in leaves as a function of copper concentration (2.0-10.0 µg L^{-1}) and exposure time (12-60 h) for *P. stratiotes*.

The activity of APX in roots rose circa threefold as compared with the activity in the leaves. Anyway, in leaves, the activity of APX was higher than control plants in practically all times of exposure and copper concentrations. This can be observed in the region darkened in the contour surface developed by model of regression in figure 12, where only exposure time linear was significant in this model.

Significant increase of APX in response to copper stress has also been reported in *C. demersum* by Devi and Prasad (1998), and *Phaseolus vulgaris* by Gupta et al. (1999). The APX activity was found to increase in the plants of *P. stratiotes* with increasing concentrations of externally supplied chromium (SINHA et al., 2005).

Srivastava et al. (2006) working with *H. verticillata* upon copper stress, related that maximum increase in activity of APX was 413% with four days at 1.6 mg Cu l^{-1}. These

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investigators observed that APX exhibited significantly high activities at all treatments while activity of CAT showed significant increase at lower copper exposures and durations. Similar results were observed for us in leaves of *P. stratiotes* to both enzymes. Srivastava et al. (2006) suggested that, high increase in activities of these enzymes could mean a breakdown of superoxide radicals by SOD to keep their level in control at the place of their generation and follow up action of APX along with CAT would have allowed plants to combat oxidative stress at least up to moderate concentrations and durations.

Sanità di Toppi et al. (2007) related that exposure to a remarkably higher level of Cd stress could have imposed to *P. stratiotes* a change in its detoxification strategy, aimed complexing in the roots as much as possible, thus preventing the translocation of potentially toxic amounts of the metal to the leaves: this would explain the conspicuous increase of phytochelatin synthesis in the roots of this species in response to 100 μM Cd, which was accompanied by a stimulation of $O_2^{\cdot-}$ by SOD and H_2O_2 by APX scavenging enzymes in these organs. In our results, in spite of not analyzing the phytochelatin synthesis, this change in the detoxification strategy could be occurring. Likewise, high concentrations of copper were observed in the roots, site where in general the responses of the antioxidant systems were relatively more pronounced including CAT, APX, GR and SOD.

The antioxidant metabolism is commonly viewed as a good indicator of the exposure to pollutants and an important protective mechanism (KANOUN-BOULÉ et al., 2009). The knowledge of how plants cope with metal-induced oxidative stress is of considerable importance in understanding the metal tolerance mechanisms evolved by plants (MONFERRÁN et al., 2009). The mechanisms by which Cu induces antioxidative responses, whether different plant species share a common defense mechanism or not, are not yet fully understood (UPADHYAY; PANDA, 2009).

Upadhyay and Panda, (2009) reported hence that, the concentration-time dependent short term copper exposure had a remarkable effect in the physiology and biochemistry, induced oxidative stress in *Pistia* plants, changes in antioxidant efficiency and ultra structural characteristics suggesting a possible mechanism of Cu phytotoxicity in water lettuce (*P. stratiotes* L.). These alterations probably are in part responsible for the decline of the submerged aquatic macrophytes vegetation, but increased antioxidant levels helped the plants to cope with the stress under repeated metal exposure.

In general, *Pistia stratiotes* is used for phytoremediation of wastewater or natural water bodies polluted with heavy metals. The species exhibit different patterns of response to Ag, Cd, Cr, Cu, Hg, Ni, Pb and Zn. A 5 mM concentration of each of these metals resulted in distinct levels of growth inhibition and biomass production in *P. stratiotes*, with almost all the elements being accumulated at high concentrations in the root system (SHAH; NONGKYNRIH, 2007).

In spite of this, the progress in phytoremediation is hindered by a lack of understanding of complex interactions in the rhizosphere and plant interactions which allow metal translocation and accumulation in plants. The evolution of physiological and molecular mechanisms of phytoremediation, together with recently-developed biological and engineering strategies, has helped to improve the capacidade of both heavy metal phytoextraction and phytostabilization (PADMAVATHIAMMA; LI, 2007).

Future studies with respect to changes in expression of major antioxidative genes along with the regulatory proteins under Cu stress can reveal better understanding of copper tolerance and toxicity in aquatic macrophytes (UPADHYAY; PANDA, 2009). Shah and Nongkynrih (2007) still affirm that, further manipulations of these genes would prove useful

to determine plant metal hypertolerance and hyperaccumulation. The strategy could be thus used as a tool to specifically select several more plant species fit for phytoremediation.

In this study, the use of Central Composite Design (CCD) represents a differential interpretation of results through the Response Surface Methodology (RSM). The CCD is advantageous since it can generate a maximum amount of information on the direct effect of test variables and their interactions while testing a minimum number of combinations (DE SCHAMPHELAERE et al. 2003). Statistical advancement in ecotoxicology is necessary to improve the guidelines in this area. In the aquatic toxicology the recent use of the factorial design has presented promising results (BAYRAKTAR, 2001; HEIJERICK et al., 2003; FALLER et al., 2003; PARK et al., 2009). In this work, the simulation of data is possible through the developed models, which together with other investigations of effect of chromium in *P. stratiotes* could be used to determinate the tolerance capacity and applied to environmental risk assessment using this species as bioindicator and biomonitor of the water ecology quality.

8.4. CONCLUSION

Central Composite Design (CCD) and Response Surface Methodology (RSM) was performed to investigate the copper-induced oxidative stress, as well as to verify changes in others biochemical parameters. The mathematic models permitted the evaluation of the differential response of enzyme's activity in relation to the exposure time and copper concentrations, and also the differential pattern response in roots and leaves. Thus, the antioxidant defense system of the *P. stratiotes* presented hyperactivity showing capacity to

tolerate the imposed stress in this sutdy, like so the antioxidant enzymes can be used as copper induced biomarkers for monitoring aquatic environments polluted with this metal. The results confirm that *P. stratiotes* can be used as a phytoremediator, reinforcing its effectiveness in copper toxicity remediation.

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CAPÍTULO 9. DYNAMIC OF AMMONIUM AND PHOSPHATE REMOVAL FROM DOMESTIC WASTEWATER USING CONSTRUCTED WETLANDS UNDER VARIOUS FLOW REGIME AND EXPERIMENTAL DESIGNS PLANTED WITH *THYPHA* SP. AND *PHRAGMITES* SP.

ABSTRACT

The use of macrophytes in constructed wetlands generally expands the capacity to remove nutrients and they are highly dynamic systems, several factors related to the operation of these complex systems can influence the processes for the purification of wastewater. This study aimed to evaluate the influence of the presence or absence of macrophytes in the capacidade of nutrient removal (ammonia and phosphate), and the capacidade of the systems being studied under different flow regimes (surface and subsurface), retention times hydraulic load and the capacity to remove nutrients. The study period comprised the summers of 2007 and 2008 and winter 2008. The results of physicochemical conditions revealed a pattern of seasonal variation. The vegetated constructed wetlands were more efficient in removing nutrients from the systems are not planted. The constructed wetlands with free floating macrophytes (FFP) and free water subsurface (FWSS) were more efficient in the removal of nutrients and loading removal of nitrogen both in the systems planted with *Typha* sp.*,* and the CWs with FWSS and subsurface flow (SSF) planted with *Phragmites* sp. and *Typha* sp. (to phosphate removal had better efficiency).The systems with subsurface flow with free water surface, showed better capacidade in removal of nutrients, related to a higher porosity and greater hydraulic retention time.

Key-words: loading rate surface, nitrogen total, porosity, subsurface flow, surface flow, time retention hydraulic.

<div align="center">RESUMO</div>

Dinâmica de remoção de amônia e fosfato de efluentes domésticos utilizando effluents domésticos sob vários regimes de fluxo e condições de operação palntados com *Typha* sp. e *Phragmites* sp.

O uso de macrófitas em wetlands construídos geralmente amplia a capacidade de remoção de nutrientes e como são sistemas extremamente dinâmicos, vários fatores relacionados à operação destes complexos sistemas podem influenciar nos processos de depuração de águas residuárias. O presente trabalho teve por objetivo: avaliar a influência da presença ou ausência das macrófitas (*Typha* sp. e *Phragmites* sp.) na capacidade de remoção de nutrientes (amônia e fosfato), bem como a capacidade dos sistemas em estudo sob diferentes regimes de fluxo (superficial e subsuperficial), tempos de retenção hidráulica e carga na capacidade de remoção de nutrientes. O período de estudo compreendeu os verões de 2007 e 2008 e o inverno de 2008. Os resultados das condições físico-químicas revelaram um padrão de variação sazonal. Os wetlands construídos plantados foram mais eficientes na remoção de nutrientes do que os sistemas não plantados. Os wetlands com macrófitas livre flutuantes (FFP) e com fluxo de água subsuperficial (FWSS) foram mais eficientes na remoção de nutrientes em ambos os sistemas plantados com *Typha* sp. E os wetlands construídos com FWSS e fluxo subsuperficial (SSF) plantados com *Phragmites* sp. e *Typha* sp., tiveram melhor eficiência para a remoção de fosfato. Os sistemas com fluxo subsuperficial com superfície de água livre, apresentaram melhor capacidade na remoção de nutrientes, relacionado a uma maior porosidade e maior tempo de retenção hidráulica.

Palavras-chave: fluxo superficial, fluxo subsuperficial, nitrogênio total, porosidade, taxa de carregamento superficial, tempo retenção hidráulica.

A constructed wetland is defined as an engineered system designed to simulate a natural wetland for waste treatment (NUTTALL et al., 1998; USEPA, 2000) and has now been successfully used in the treatment of several wastewaters such as domestic sewage, urban runoff and storm water, industrial and agricultural wastewater, and leachate (SCHOLZ; LEE, 2005). Although considerable progress has been made in our understanding of the physical, chemical and biological processes that facilitate treatment, inconsistent results suggested that further research is needed to optimize system functioning (KADLEC; KNIGHT, 1996; KARATHANASIS et al., 2003; TAO et al., 2006; LEE et al., 2009). Constructed wetlands are classified into two major types according to hydraulic water flow characteristics in the system: surface flow (SF) and subsurface flow (SSF) systems (VYMAZAL, 2007). Currently, some researchers are developing new wetland systems and the selection of the most appropriate one depends on the targeted pollutants, the available land, and the acceptable level of maintenance and management (LEE et al., 2009).

In general, the constructed wetlands are used for purification of secondary-treated wastewater, and a wide range of nutrient removal efficiencies was reported (FRASER et al., 2004; GREENWAY, 2005), although nitrogen (N) and phosphorus (P) removal is known to be somewhat problematic (BRIX et al., 2001). Numerous environmental factors can influence the removal of nitrogen and phosphorus, including temperature, hydraulic retention time (HRT), type and density of vegetation, the characteristics of microbial communities, climate, the distribution of wastewater and influent characteristics, etc (GEARHEART, 1992; ZHANG et al., 2008).

Macrophytes also play an important role in wastewater treatment through uptake of nutrients, surface bed stabilization, and other mechanisms (GERSBERG et al., 1986; KADLEC; KNIGHT, 1996; HUETT et al., 2005; ZHANG et al., 2008). Wetland systems with vegetation typical remove greater amounts of total nitrogen than non-vegetated systems (COLEMAN et al., 2001; YANG et al., 2001; HUETT et al., 2005). Common macrophytes used in constructed wetlands are reed (*Phragmites australis*) and cattail (*Typha* spp.), all characterized as water-tolerant macrophytes that are rooted in the soil but emergent above the water surface (COLEMAN et al., 2001; VYMAZAL, 2005). Macrophytes also serve to stabilize the bed surface, increase the porosity throughout the wetland volume, absorb and store nutrients, prevent channelized flow, etc (TANNER; SUKIAS, 1995). In gravel-bed constructed wetlands in which emergent plants are rooted, the gravel substratum, in addition to providing physical support for plant growth, and surfaces for sorption and biofilm growth, promotes the settling and filtration of suspend solids (TANNER; SUKIAS, 1995). The gravel occupies a considerable proportion of the bed volume in these systems, generally leaving an interstitial void space of between 30 and 45%. Growth of biofilms and plant root systems within these void spaces further reduce their effective interstitial volume (TANNER et al., 1998). As the gravel matrix gradually clogs, both its hydraulic conductivity and the effective residence time of wastewaters flowing through it are reduced, affecting flow pathways and ultimately the treatment capacidade of the wetland (TANNER; SUKIAS, 1995).

Therefore, further investigations are needed to clarify optimal design options for these parameters in the treatment process, and evaluate the sustainable removal capacidade by monitoring the water quality. In the present study, constructed wetlands (CW) based-gravel with surface flow (SF) and subsurface flow (SSF) were established to receive the wastewater treatment. The treatments included CW with and without vegetation, wetlands with

hydroponic systems with floating macrophytes (FFP), and systems with free water surface (FWS) and subsurface (FWSS), and horizontal subsurface flow (HSSF or SSF). The objectives of this work were to (1) comparing in ammonium and phosphate removal capacidade in the CWs submitted in the different flows (SF and SSF); (2) comparing the nutrient removal associated to changes in hydraulic retention time and porosity in wetlands with gravel-bed; (3) investigating the performance of emergent macrophytes (*Thypha* sp. and *Phragmites* sp.) in the wetland systems for the nutrient removal.

9.2. MATERIAL AND METHODS

The system used in the provincie of León (NW Spain) treats part of the urban wastewater generated by a conventional wastewater treatment plant that consist of a primary treatment (screening, sand removal, fat removal and primary clarifier) and a secondary treatment (plug-flow activated sludge with nitrification/denitrification and secondary clarifier). The plant was designed to treat the wastewater of 330,000 equivalent inhabitants with an inflow of 123,000m^3 d^{-1} and an HRT of about 6 h.

Experimental system

In the present study, sampling was done in site adjacent the unit center of water treatment of province with eight containers (0.50 x 1.00m^2). The experimental units consisted: CW1 and CW5, hydroponic systems planted with *Typha* sp. and *Phragmites* sp., respectively

without substrate and surface flow (HS floating); CW2, free water surface flow (FWS) with gravel until 0.25 m planted with *Typha* sp.; CW3, free water subsurface flow (FWSS) with gravel until 0.25 m planted with *Typha* sp.; CW4, free water subsurface flow (FWSS) with gravel until 0.25 m unplanted; CW6, horizontal subsurface flow gravel-filled planted with *Phragmites* sp. with threshold loading rate; CW7, horizontal subsurface flow gravel-filled planted with *Phragmites* sp.; CW8, horizontal subsurface flow gravel-filled unplanted according to Figure 1.

Sampling regime

Throughout the operation period, the load of nitrogen to the whole wetland system has been 14.6 ± 5.3 mg L^{-1}. The experiment was carried out from August 2007 to September 2007 (Summer 2007), January 2008 to March 2008 (Winter 2008) and June 2008 to August 2008 (Summer 2008). Grab samples were taken once a week for each period, always on the same day and at the same time. Amber glass bottles were used to collect 1-litre samples, which were transported refrigerated to the laboratory, where they were processed in less than 24 h.
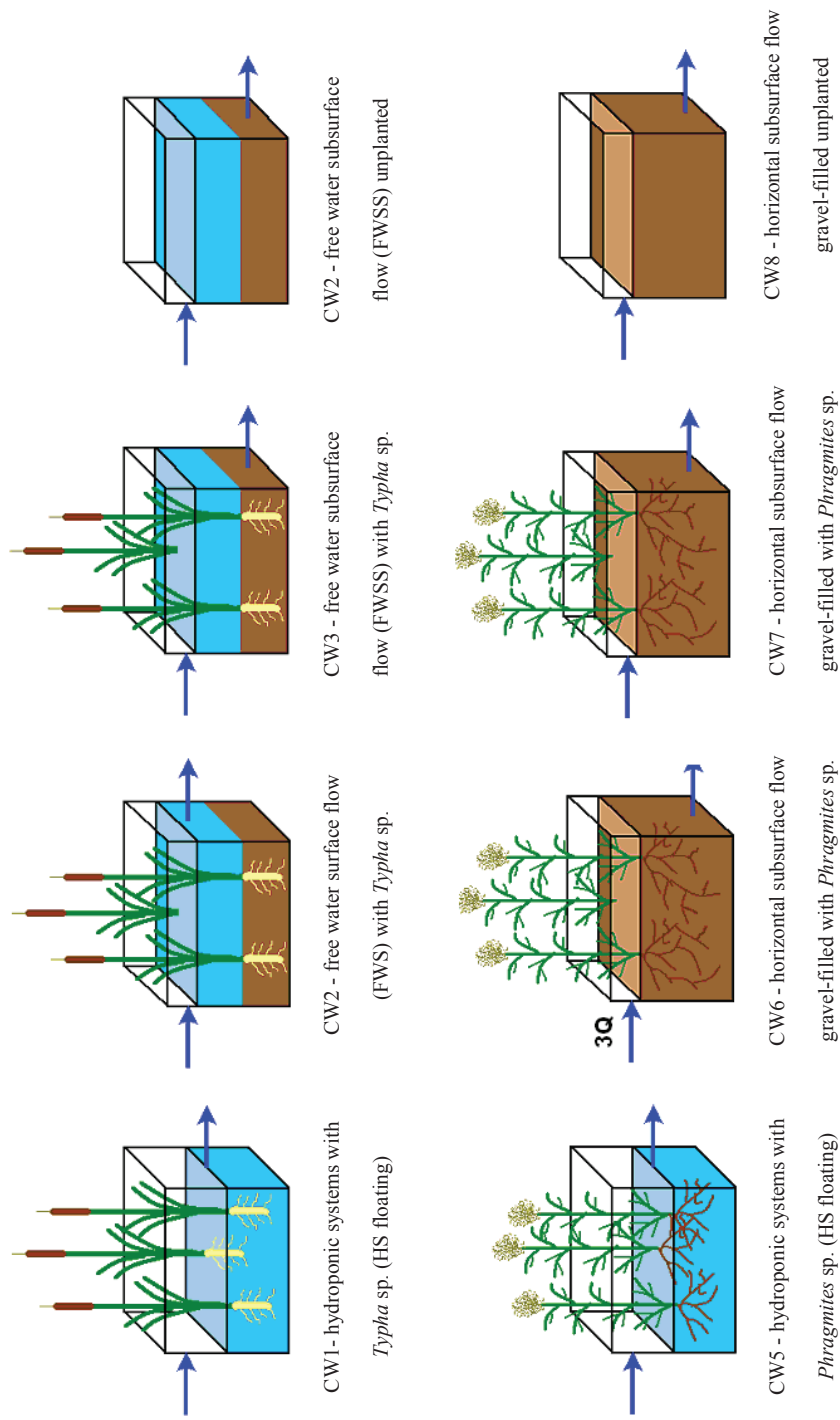
Figura 1. Schematic representation of the constructed wetlands experimental structure (WC) with and without macrophytes (*Typha* sp. e *Phragmites* sp.) under flow regime: HS, horizontal surface; FS, surface flow; FSSH, horizontal subsurface flow).

Water quality parameters

The same time, 1000 ml water samples from influent and effluent of each of the systems were collected in plastic bottles for chemical analysis. The total Kjeldahl nitrogen (TKN) and total phosphorous (TP) were analyzed immediately according to the APHA (1989) recommendations (methods: 5220 A, 5210 B, 4500 N-org B, 4500-P B and E, respectively). Temperature, pH, conductivity and dissolved oxygen (OD), and redox potential were measured in situ with portable electrodes (WTW).

Statistical analysis

Statistical procedures were carried out using the Statistical (Statsoft 7.0). The normality of the variables was verified to support the use of parametric or nonparametric tests. Otherwise, datawere either log-transformed (superficial removal rates) or arcosin-transformed (percentages). To compare seasonal efficiencies, Mann–Whitney U test ($p < 0.05$) was applied to each treatment database. One-way ANOVA analysis was used to evaluate the signification of the differences founded between treatment systems. Once verified the variances dissimilarity, the Tukey test post hoc comparison was used to test all pairwise of treatments data averages ($p < 0.05$ or $p < 0.01$).

Constructed wetlands and wastewater characterization

The average results and standard deviations of pH, dissolved oxygen (DO) and redox potential (E_H) in the inflow and outflow of the constructed wetlands which received wastewater are presented in Table 1. Water flowing through the wetlands has a pH near neutrality, and the change of water pH was of insignificant magnitude. The unplanted system showed slightly higher pH values than the planted ones, both in the summer/07 and winter/08. The seasonal differences in the unplanted systems (CW4 and 8) were not significant, but in the planted ones (CW3 and 7) those differences were relevant ($p < 0.01$). Influent pH remained relatively well buffered, with values between 7.1 and 7.4. The pH differences between surface and bottom were significant only to the 2, 4 and 8 CWs.

Table 1. Mean values and standard deviations (in parentheses) of pH, dissolved oxygen, and redox potential of the constructed wetland (CW) in the surface (s) and the bottom (b) depth during samplings period[#].

	pH (units)			DO (mg L^{-1})			E$_H$ (mV)			Statistic
	S/07	W/08	S/08	S/07	W/08	S/08	S/07	W/08	S/08	
CW1 s	6.7(0.09)^{**a}	6.7(0.07)^{**a}	6.9(0.14)^{**b}	2.0(0.59)^{*a}	0.7(0.45)^{*a}	0.5(0.46)^{**b}	272.8(71.05)^{**b}	22.4(131.10)^{**a}	36.0(151.08)^{**a}	aaa **
CW1 b	6.7(0.13)^{**a}	6.6(0.09)^{**a}	6.9(0.13)^{**b}	2.0(0.54)^{*a}	0.4(0.20)^{*a}	0.7(1.53)^{**b}	260.6(50.41)^{**b}	-24.7(110.31)^{**a}	-61,08(139.00)^{**a}	aba **
CW5 s	7.0(0.08)^{**a}	7.0(0.14)^{**a}	6.6(0.16)^{**b}	1.6(1.04)^{*b}	0.6(0.59)^{**a}	0.8(0.49)^{**a}	177.5(42.92)^{**b}	-10.2(105.72)^{**a}	47.6(140.46)^{**a}	aaa *
CW5 b	7.0(0.05)^{**a}	7.0(0.14)^{**a}	6.6(0.17)^{**b}	1.7(0.52)^{**b}	0.5(0.55)^{**a}	0.5(0.34)^{**a}	81.4(71.15)^{**b}	-50.1(100.92)^{**a}	-25.2(131.63)^{**a}	abb *
CW2 s	7.1(0.06)^{*a}	7.0(0.11)^{**b}	7.3(0.17)^{*a}	3.1(1.06)^{**b}	0.6(0.28)^{**a}	0.6(0.38)^{**a}	302.5(38.67)^{**b}	-10.4(98.20)^{**a}	62.6(130.52)^{**a}	aaa **
CW2 b	6.9(0.13)^{*a}	6.5(0.06)^{**b}	6.9(0.22)^{*a}	0.5(0.56)^{**b}	0.6(0.50)^{**a}	0.3(0.35)^{**a}	109.2(70.47)^{**b}	-2.6(133.56)^{**a}	-100.9(120.50)^{**a}	bbb **
CW3 s	7.0(0.04)^{*a}	7.1(0.16)^{**b}	7.4(0.20)^{**c}	2.6(1.03)^{**b}	0.7(0.39)^{**a}	0.5(0.31)^{**a}	239.5(72.79)^{**b}	14.5(94.89)^{**a}	9.0(130.13)^{**a}	ns
CW3 b	6.9(0.04)^{*a}	6.9(0.24)^{**b}	7.3(0.30)^{**c}	0.5(0.66)^{**b}	0.4(0.18)^{**a}	0.3(0.28)^{**a}	166.6(74.09)^{**b}	-16.1(96.56)^{**a}	-55.1(120.00)^{**a}	ns
CW4 s	8.1(0.33)^{ns}	7.8(0.56)^{ns}	8.3(0.85)^{ns}	11.11(4.02)^{**b}	3.5(3.63)^{**a}	2.4(2.46)^{**a}	276.0(44.94)^{**a}	39.8(122.39)^{**ab}	25.4(130.60)^{**b}	aaa **
CW4 b	7.4(0.21)^{ns}	7.5(0.58)^{ns}	7.4(0.27)^{ns}	0.4(0.21)^{**b}	1.2(1.94)^{**a}	0.3(0.27)^{**a}	-12.6(36.26)^{**a}	3.2(126.66)^{**ab}	-118.1(141.44)^{**b}	bbb **
CW6 s	6.7(0.05)^{*a}	6.9(0.16)^{**c}	6.6(0.24)^{*b}	1.4(1.22)^{**b}	0.6(0.32)^{**a}	0.4(0.33)^{**a}	153.0(59.76)^{*b}	-2.6(102.52)^{*a}	-14.7(85.80)^{*a}	ns
CW6 b	6.8(0.10)^{*a}	7.0(0.13)^{**c}	6.8(0.78)^{*b}	0.6(0.66)^{**b}	0.4(0.14)^{**a}	0.3(0.23)^{**a}	20.2(96.87)^{*b}	-47.2(121.48)^{*a}	-31.5(83.83)^{*a}	ns
CW7 s	6.7(0.08)^{**a}	6.9(0.08)^{**b}	6.5(0.06)^{**c}	0.8(0.30)^{*b}	0.6(0.33)^{*a}	0.6(0.49)^{*a}	164.5(64.51)^{*b}	38.8(89.73)^{*a}	25.9(103.00)^{*a}	ns
CW7 b	6.7(0.08)^{**a}	6.9(0.07)^{**b}	6.5(0.24)^{**c}	0.3(0.30)^{*b}	0.3(0.15)^{*a}	0.4(0.38)^{*a}	87.7(78.12)^{*b}	24.1(98.75)^{*a}	22.1(107.88)^{*a}	ns
CW8 s	7.5(0.08)^{ns}	7.4(0.13)^{ns}	7.4(0.10)^{ns}	0.9(0.39)^{**a}	0.70(0.47)^{*a}	0.3(0.26)^{**b}	83.8(20.33)^{*a}	46.2(87.50)^{*a}	-6.5(128.91)^{*b}	aaa **
CW8 b	7.5(0.06)^{ns}	7.4(0.13)^{ns}	7.3(0.16)^{ns}	0.2(0.09)^{**a}	0.4(0.15)^{*a}	0.3(0.28)^{**b}	-11.0(37.37)^{*a}	11.1(108.31)^{*a}	-91.4(147.17)^{*b}	bbb **
Influent	7.3(0.15)	7.1(0.07)	7.4(0.14)	1.3(0.68)	0.7(0.28)	0.4(0.24)	109.8(70.94)	0.46(114.94)	26.4(165.84)	

[#]S/07, samplings from August 2007 to September 2007 (n = 8); W/08, samplings from January 2008 to March 2008 (n = 10) and S/08, samplings from June 2008 to September 2008 (n =13).

* In the line, significant differences to 95% of confidence level; and ** significant differences to 99% of confidence level, these by compare differences of seasons.

* In the column, significant differences to 95% of confidence level; and ** significant differences to 99% of confidence level, these by compare differences of depth, being the first letter represents the pH, the second letter represents the DO and the third letter represents the E$_H$.

The change of DO concentrations were very similar; in the wetlands the effluent ranged from 0.3 to 3.1 mg L^{-1} for planted systems, from 0.2 to 11.1 mg L^{-1} to unplanted systems, and 0.4 to 2.0 mg L^{-1} for hydroponic systems with floating macrophytes (Table 1). In general, the values of DO concentrations were less than 1.0 mg L^{-1} in all wetlands and all seasons, with exception of the values of DO concentrations in the CW4, CW1, CW5 and CW6. The highest values were observed in the CW4 surface varying from 2.4 to 11.1 mg L^{-1}. DO concentrations were higher in the free water surface (FWS), and sub-surface flow (FWSS) wetland in the summer/2007, whereas the values of DO concentrations were less than 1.0 mg L^{-1} in the subsurface flow (SSF). The occurrence of seasonal differences were observed in the 2 and 8 CWs, where in the summer/08 it was different in relation to the other seasons; (for too much CWs the summer/07 was different). Those differences were significant ($p<0.01$) and for CW7 significant at $p<0.05$. In the 3, 6 and 7 CWs the differences between surface and bottom were not significant ($p<0.01$) and in the CW5 the difference was significant at $p<0.05$. There were significant differences ($p<0.01$) between surface and bottom in the 1, 2, 4, 5 and 8 CWs to DO concentrations.

Redox potential fluctuated from ca. -10 to +300mV (summer/2007), -50 to +46mV (winter/2008) and -118 to +62mV (summer/2008). Seasonal variations were observed in E$_H$, with differences in the depth, where higher values of E$_H$ occurred in the surface in relation to the bottom (Table 1). Based on the approximate zones scheme that corresponds to aerobic (E$_H$ > +300mV), anoxic (+300mV > E$_H$ > +100mV) and anaerobic (E$_H$ < +100mV) conditions proposed by Gambrell and Patrick (1978), in the summer/2007 the values reflected a condition anoxic in the 1 to 5 CWs, with exception of the media E$_H$ in the CW2 surface that was aerobic (302.5 mV). In the 6 to 8 CWs the anaerobic conditions were predominant. The anaerobic condition prevailed during entire winter and summer/08 in almost all CWs in the

surface, being anoxic in the bottom only of CW2 and 4. In general, the E_H average values in the summer/07 showed significant seasonal differences ($p<0.01$) in relation to winter and summer/08, but these differences for the 6 and 7 CWs were significant at $p<0.05$. To the E_H unplanted systems in the summer/08 there was some difference in relation to other studied seasons, being this difference significant at $p<0.01$ to CW4 and $p<0.05$ to CW8. The E_H differences between surface and bottom were significant only to the 2, 4, 5 and 8 CWs.

Removal rates of Nitrogen

Influent wastewater and effluent concentrations of ammonium varied seasonally (Figure 2). The average value of influent ammonium was 14.6 ± 5.3 mg L^{-1}. In the hydroponic floating systems (FFP), with the *Typha* sp. (CW1) they showed a better capacidade than with *Phragmites* sp. (CW5), maintaining ammonium concentrations smaller with exception of the months July and August/2008. The average values of ammonium effluent to CWs (Figure 1) were 5.6 ± 4.9 (mg L^{-1}) for the *Typha* sp. (CW1) and 8.7 ± 9.2 (mg L^{-1}) for the *Phragmites* sp. (CW5).

Comparing the two types of flow applied in CW2 and CW3, both planted with *Typha* sp., the behavior was very similar in the summer/2007, but the CW3 with free water subsurface flow was superior in all the period, studied reducing effluent nitrogen concentration. The percentage of nitrogen removal (Figure 3) was bigger at the wetlands with FWSS (CW3) than with FWS (CW2), and the difference was not only significant in the summer/08 (Table 2).
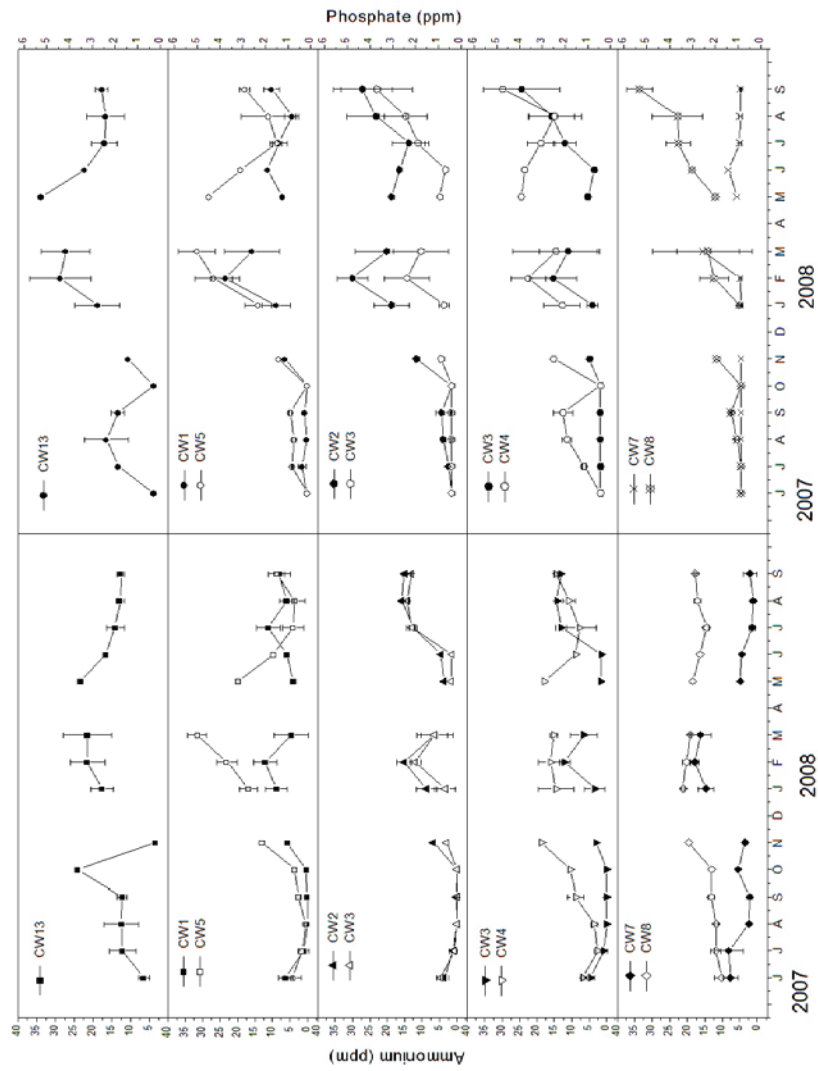
Figure 2. Comparison of influent (CW13) and effluent concentration-time average curves for CWs during the sampling period.

Average ammonium concentration in almost all period for CWs planted were lower than for unplanted, exception in July and August/2008 in the CW4 where the concentration was higher. Effluent average values to ammonium (Figure 2) were 7.5 ± 6.5 (ppm) for the *Typha* sp. (CW3); 10.0 ± 5.5 (ppm) for the unplanted wetland (CW4); and 6.7 ± 6.3 (ppm) for the *Phragmtes* sp. (CW7); 15.8 ± 3.7 (ppm) the unplanted wetland (CW8). The average percentages of reduction in relation to ammonium influent were: 51.4% to CW3, 68.5% to CW4, 45.8% to CW7. While, there was an increase of 8.2% for the CW8. Comparing the average percentage of reduction of the ammonium concentration between the planted and unplanted system. The free water surface flow wetland planted with *Typha* sp. (CW3) showed 25% reduction in relation to CW4, and free water sub-surface flow wetland planted with *Phragmites* (CW7) presented a reduction of 57.4% in relation to CW8. Likewise, the CWs pairs that compare planted and unplanted systems (3-4 and 7-8), both maintained similar behavior. During the experimental period, *Typha* sp. had effluent loading with smaller nitrogen concentrations, with exception of the summer/08 when *Phragmites* sp. was more efficient in the ammonium removal.

Table 2. Sumary of statistic analysis of nutrient and volumetric loading removed comparing different CWs from samplings in the summer 2007-2008 and winter 2008.

CW	Nutrient removed (%)						Volumetric loading removed (g m^{-3} dia^{-1})					
	Summer/07		Winter/08		Summer/08		Summer/07		Winter/08		Summer/08	
	F value	p	F	p	F value	p	F value	p	F	p	F	p
1-5	**8.4389**	**0.00229**	**8.1856**	**0.00104**	**7.2175**	**0.00081**	0.30768	0.81943ns	**9.6293**	**0.00072**	4.2680	0.01613*
2-3-4	**8.1888**	**0.00000**	**7.3835**	**0.00000**	1.3506	0.23487ns	4.9470	**0.00079**	**8.5889**	**0.00002**	**9.9758**	**0.00000**
2-3	4.6756	0.01892*	**8.0837**	**0.00111**	0.97874	0.44025ns	6.2908	**0.00825**	**11.381**	**0.00030**	**23.432**	**0.00000**
2-4	**8.0817**	**0.00271**	**10.617**	**0.00028**	0.82291	0.52520ns	8.9821	**0.00215**	**14.619**	**0.00008**	**15.453**	**0.00001**
3-4	**14.050**	**0.00027**	**7.6664**	**0.00143**	1.7246	0.18221ns	1.3340	0.30935ns	2.3937	0.10652ns	2.0766	0.13248ns
7-8	**33.256**	**0.00000**	1.9478	0.15465ns	368.07	**0.00000**	24.133	**0.00002**	7.0363	**0.00313**	109.58	**0.00000**
3-7	**8.2211**	**0.00254**	**18.040**	**0.00001**	22.355	**0.00000**	0.20266	0.89256ns	7.9224	**0.00184**	19.603	**0.00000**
4-8	**23.036**	**0.00003**	**11.317**	**0.00020**	6.7355	**0.00119**	20.896	**0.00005**	7.0363	**0.00313**	15.776	**0.00001**

Values in bold are significantly different at $P < 0.01$, and values with asterisk are significantly different at $P < 0.05$, and values with ns are not significantly different at $P < 0.05$.

The average values for the planted systems were significantly ($p<0.01$) different from the unplanted ones, what indicates that the role of the aquatic plants in CWs could have a good capacidade in nitrogen removal in wastewater treatment.
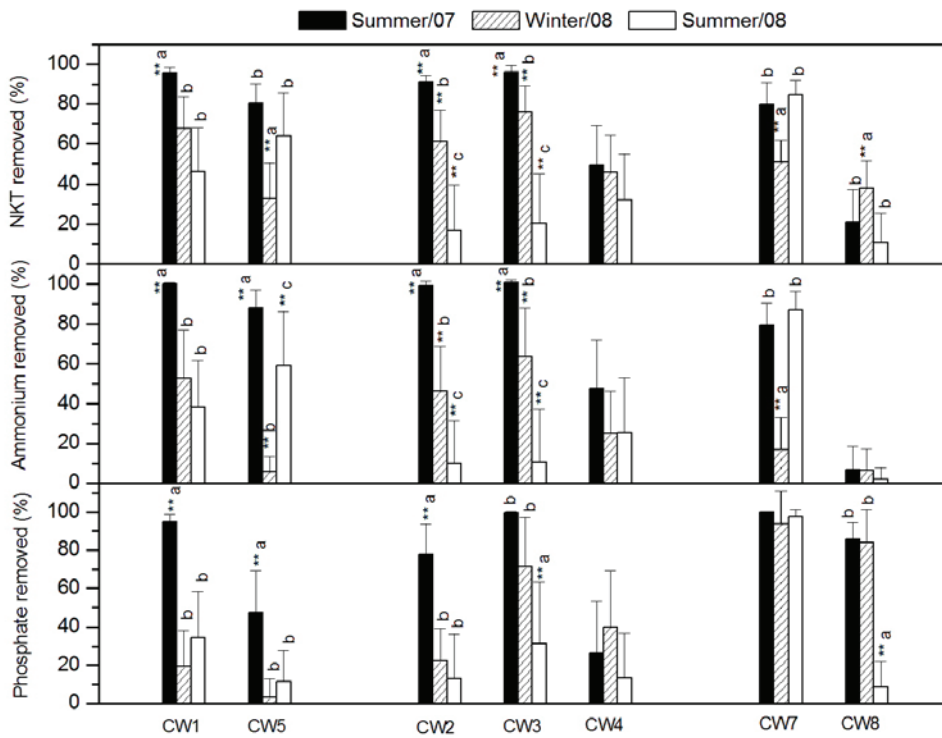

Figure 3. Average values of nutrient removed (%) in the wetlands studied during the sampling period. The asterisk represent the percentage of significance at $p < 0.01$ (**) and $p < 0.05$ (*).

Nutrients (ammonium and NTK) removal was generally higher in the 1 to 5 constructed wetlands in the summer/07 (Figure 3). The *Typha* sp. was more efficient in the nutrients removal at the CW1 than CW5 (both in hydroponic floating system), in almost all the period of study, but in the summer/08, the *Phragmites* sp. was more efficient on the nitrogen removal, both significantly different to $p<0.01$ (Table 2). Planted wetlands showed greater removal forms of N of wastewaters than unplanted, the average percentage of

ammonium and NTK removal, respectively, was: 58% and 62% in the summer/07, 28% and 53% in the winter/08, and 31% and 32% in the summer/08. The nutrients removal in CW3 planted with *Typha* sp. during all seasons was bigger if comparing planted to unplanted systems on free water sub-surface flow (CW3xCW4), being unsignificant ($p<0.01$) only in the summer/08. The nitrogen removal was not significant in the summer/08 (Table 2). The constructed wetlands on sub-surface flow and planted with *Phragmites* sp. showed an increased nutrients removal during the experimental period in relation to unplanted systems (CW7xCW8). Those differences were significant to 95% and 99% in the summer/2007 and 2008, respectively, and were not in the winter/2008. There was a decline in removal of nitrogen with the time of maturation of the 2 and 3 CWs; that behavior was demonstrated also to CW4, but only in the NTK removal. There was significant difference (Table 2) between the unplanted systems (CW4 and CW8) in the removal of nitrogen, which had the lowest percentage of removal. The CW8 showed the smallest percentages, 5.2% to ammonium and 23.3% to NTK. To compare the 3 and 7 CWs, *Typha* sp. (CW3) demonstrated superior percentages of the nitrogen removal but it decreased with passing of time. Conversely of what occurred with *Phragmites* sp. (CW7), this specie kept elevated percentages during the seasons, excepted only for the winter/2008. Those differences were significant to $p<0.01$ (Table 2).

Removal rates of Phosphate

Influent wastewater concentrations of phosphate varied seasonally (Figure 1). Similarly to outflow nitrogen concentration, the average outflow phosphate concentrations in the entire period for planted CWs were also lower than for the unplanted. The average values

to CWs phosphate concentrations (Fig. w) were 0.9 ± 0.9 mg L^{-1} to *Typha* sp. (CW3) and 0.1 ± 0.3 to *Phragmites* sp. (CW7) and 1.5 ± 0.8 and 1.0 ± 1.0 mg L^{-1} for the unplanted wetlands CW4 and CW8, respectively. Aside from that, the average values inflow wastewater was 2.1 ± 1.0 mg L^{-1}. The media percentages of reduction were 57% to CW3, 29% to CW4, 95% to CW7, 52% to CW8. *Typha* sp. was the more efficient in reducing outflow phosphate concentration in relation to *Phragmites* sp., this species, during the period of this study, presented depleted efficiency to outflow phosphate removal, if compared constructed wetlands on hydroponic systems with floating macrophytes (CW1 and CW5). In all the period of study, it was observed that *Phragmites* sp. was more efficient in planted and unplanted systems. In winter/2008, both species were inefficient in the reduction outflow phosphate concentration. Conversely the behavior to ammonium removal, CW2 and CW3 both planted with *Typha* sp., which differred in his type of flow, showed great oscillation during the period of study and were more efficient in the CW3 at the phosphate removal.

The percentage of removal phosphate (Figure 3) for wetlands planted on FWSS (CW3) was significant higher in relation to unplanted ones (CW4), increasing from 25% to 100% (summer/07, $P < 0.05$). However, it had a less pronounced increase, varying from 40% to 60% in the winter/08 ($p<0.01$) and from 13% to 30% in the summer/08 (in this season, no significant, Table 2). In this case, the efficiency was decreasing during the period of study, but to the 7 and 8 wetlands the capacity of removal was maintained in almost all the experimental period, there was a reduction just in the summer/2008 (Figure 3). The average of phosphate removal was greater in the wetland planted with *Phragmites* (CW7), if compared with *Typha* sp. (CW3), and the differences were significant $P < 0.01$ (Table 2) in all seasons. The analysis of phosphorus in plants showed 7% of build up in *Phragmites* in relation to *Typha* sp. (not showed data). The comparison between unplanted systems CW4 and CW8, the wetland on

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sub-surface flow (CW8) demonstrated better capacidade to the phosphate removal than CW4, and the differences were significant to $P < 0.01$(Figure 3 and Table 2). Likewise to nitrogen removal (ammonium and NTK), the FWSS (CW3) showed better results than FWS (CW2), both planted with *Typha*.sp. It was not significant only in the summer/2008.

Hydraulic Conditions of Wetlands: loading rate, porosity, hydraulic retention time and harvesting of plants

The volumetric and superficial loading removals to nitrogen (ammonium and NTK) are presented in the Figure 4 and Table 3. The inflow used in constructed wetlands was the low hydraulic loading rate < 1 gNH_4-N m^{-2} d^{-1}. In relation to volumetric loading it is possible those analysis did not show great differences between CW1 and 5 because it is the same theoretical water volume of wetlands. Besides, in the summer/2007 and winter/2008 the volumetric loading removals of nitrogen were greater in CW1 than in CW5. That could be occurring because the difference between theoretical water volume (225.92 L to both wetlands) and measured water volume (121.92 L to CW1 and 79.62 L to CW5) demonstrated that there is a bigger root volume at the CW1, and consequently, it conducted a decrease of porosity. Thus, analyzing the hydraulic retention time (HRT) in both wetlands in all period of study, in CW1 HTR was 2.8 times smaller than in CW5. Similarly, the volumetric and superficial loading removals to phosphate were greater in CW1 than in CW5, but on the other hand, regarding to loading rate removal phosphate in the winter/2008 was smaller and presented negative values indicative of organic matter production.
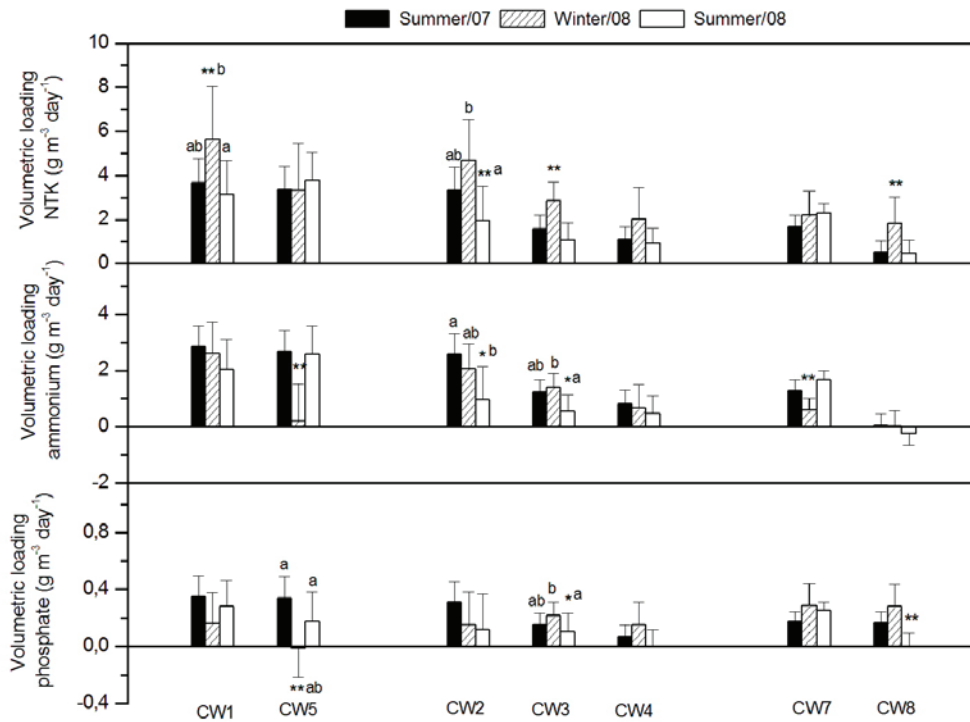
Figure 4. Average values of volumetric loading removal of nutrients (g m^{-3} day^{-1}) in the wetlands studied during the sampling period. The asterisk represents the percentage of significance at P< 0.01 (**) and P < 0.05 (*), and the different letters represent significant difference among seasons.

While comparing wetlands CW2 and 3, both showed increase loading volumetric and superficial loading removals mainly in the winter/2008, except for volumetric loading in the CW2, where the rate of loading of ammonium was decreased by the time of study.

Table 3. Mean values and standard deviations (±) of loading removal in constructed wetlands (CW[a]) during sampling period[b].

Constructed Wetlands	Loading removal (g m⁻² dia⁻¹)								
	Summer/07			Winter/08			Summer/08		
	Phosphate	Ammonium	NKT	Phosphate	Ammonium	NKT	Phosphate	Ammonium	NKT
CW1	0.08 ± 0.03	0.64 ± 0.17	0.84 ± 0.24	0.04 ± 0.05	0.59 ± 0.26	1.27 ± 0.55	0.06 ± 0.04	0.46 ± 0.25	0.71 ± 0.34
CW5	0.08 ± 0.03	0.60 ± 0.17	0.76 ± 0.23	0.00 ± 0.05	0.05 ± 0.30	0.75 ± 0.47	0.04 ± 0.05	0.58 ± 0.23	0.85 ± 0.28
CW2	0.08 ± 0.04	0.63 ± 0.19	0.82 ± 0.26	0.04 ± 0.06	0.51 ± 0.22	1.15 ± 0.47	0.03 ± 0.06	0.24 ± 0.29	0.48 ± 0.39
CW3	0.07 ± 0.03	0.55 ± 0.20	0.72 ± 0.27	0.10 ± 0.04	0.64 ± 0.21	1.30 ± 0.37	0.05 ± 0.06	0.25 ± 0.26	0.49 ± 0.35
CW4	0.03 ± 0.04	0.36 ± 0.21	0.49 ± 0.26	0.07 ± 0.07	0.29 ± 0.38	0.90 ± 0.65	0.00 ± 0.05	0.21 ± 0.28	0.41 ± 0.31
CW6	0.22 ± 0.11	0.90 ± 0.66	1.32 ± 0.86	0.02 ± 0.20	-0.22 ± 0.52	1.89 ± 1.04	0.04 ± 0.20	0.30 ± 0.75	0.95 ± 0.85
CW7	0.08 ± 0.03	0.55 ± 0.17	0.72 ± 0.23	0.12 ± 0.07	0.25 ± 0.19	0.96 ± 0.46	0.11 ± 0.03	0.72 ± 0.14	1.00 ± 0.18
CW8	0.07 ± 0.03	0.02 ± 0.16	0.20 ± 0.21	0.11 ± 0.06	0.01 ± 0.21	0.71 ± 0.47	0.00 ± 0.04	-0.09 ± 0.17	0.17 ± 0.25

[a]CW1: *Typha latifolia*, hydroponic systems with floating macrophytes (FFP); CW5: *Phragmites australis*, in FFP (equal previous); CW2: *Typha latifolia* free water surface (FWS); CW3: *Typha latifolia*, free water subsurface (FWSS); CW4: free water surface unplanted (FWSS); CW6: *Phragmites australis*, subsurface flow (SSF) with HRL (x3); CW7: *Phragmites australis*, subsurface flow (SSF); CW8: subsurface flow unplanted (SSF).

[b]S/07, samplings from August 2007 to September 2007 (n = 8); W/08, samplings from January 2008 to March 2008 (n = 10) and S/08, samplings from June 2008 to September 2008 (n =13).

A similar behavior was observed to volumetric and superficial loading removals phosphate in those wetlands. In this case, there was at CW2 occupied volume of plants stem (zone 1) an increasing of 60% in relation to CW3, which represents a reduction of 21% of porosity in this wetland. In zones 2 and 3 the porosity in the CW3 showed to be about 14% bigger. There were differences between the HRT mainly in the summer/2007, where the average value was 5.7 to CW2 and 7.7 to CW3, while the average values in winter and summer/2008 were of 4.1 and 3.4 respectively. Another difference, despite of the harvest of plants between those wetlands, was the dry weight of plants: 20.6 kg in CW2 and 12.5 kg in CW3.

In the case of the CW3, 4, 7 and 8 wetlands while comparing the planted and unplanted systems for volumetric and superficial loading removals to nitrogen and phosphate, the behavior was similar (Figure 4 and Table 3). The planted systems showed more elevated loadings than the unplanted ones. The lowest values of loadings ammonium were observed in CW8 in all seasons. Probably, the presence of plants in CW3 and 7 was the responsible by the increase of, in media, 16% of porosity in zones 2 and 3 in relation to 4 and 8 CWs, respectively. In these wetlands, there was a difference in relation to hydraulic retention time (HRT); the average values for each season were to 3 and 4 CWs: 7.7 and 5.2 days (summer/2007), 4.4 and 5.7 days (winter/2008), 3.5 and 4.4 days (summer/2008), respectively.

The volumetric and superficial loading removal to nitrogen for wetlands 3 and 7 was similar, but the loading removal to phosphate was better in the CW7. In comparing those wetlands the volume occupied by roots and gravel (zone 2) in CW3 was 21% lower than in CW7, and in zone 3 the increasing was of 6%. In relation to the HRT, there was a great difference in these wetlands, where the average in the period of study was 4.4 days to CW3

and 0.9 days to CW7. The dry weight of plants harvest showed an increase of 129% to CW3 in relation to CW7. The volumetric and superficial loading removal to nitrogen was superior in CW4 in relation to CW8; the inverted relation was observed to loading removal to phosphate. In wetlands 4 and 8, both unplanted with free water sub-surface and sub-surface flow, respectively, showed differences in their porosity, where in CW8, the porosity was in media 16% higher in the zones 2 and 3 than in CW4. Likewise, the differences observed in HRT in the wetlands 3 and 7 and in wetlands 4 and 8, were 5.1 days and 0.9 days, respectively.

9.4. DISCUSSION

Constructed wetlands and wastewater characterization

The means of pH and DO concentrations observed were within the broad ranges that the results generally reported in the literature (TANNER et al., 1999; COLEMAN et al., 2001; IAMCHATURAPATR et al., 2007; LIN et al., 2008;), working with similar systems. Planted systems showed the lower levels of DO concentrations when compared with unplanted treatment (IAMCHATURAPATR et al., 2007). However, in most systems designed for the treatment of domestic or municipal sewage the supply of dissolved organic matter is sufficient and aerobic degradation is limited by oxygen availability (VYMAZAL, 2005). The aerobic degradation of planted materials inside constructed wetland can be consuming an amount of O_2 in comparison with unplanted systems. Otherwise the plant above-ground biomass limited a contact between atmospheric phase and water phase, resulting in low O_2 transfer from air to the water. Contrary to the unplanted systems, that are freely open-surface systems, and a

contact between the atmospheric phase and the water one was larger than the planted systems, resulting in high O_2 transfer from air to water. These are any reasons which could explain this phenomenon (GOPAL, 1999; KYAMBADDE et al., 2004; THULLEN et al., 2005). It has also been reported by many studies that planted treatments not only improved the DO concentration of treated water, but also displayed positive results over unplanted systems (URBANC-BERCIC, 1994; COLEMAN et al., 2001; HAM et al., 2004).

Redox potential is a good indicator for the state of the reed bed, which ultimately results from the accumulation of a large number of different influencing parameters (KAYSER et al., 2002). The predominance of anaerobic conditions in this study could be explained because, in subsurface flow wetlands, the limited contact of the wastewater with the atmosphere coupled with the high biological oxygen demand of the influent wastewater stream results in anaerobic conditions predominant throughout the water column (COLEMAN et al., 2001). Although the measurement of E_H has widely been used to characterize oxidation-reduction conditions in wetland soils, its interpretation is associated with a lot of uncertainty in the exact sense of chemical science. Dušek et al. (2008) suggested that vegetated treatment beds of subsurface horizontal flow constructed wetlands can be extremely dynamic systems.

Nitrogen

Removal of N occurred by plant uptake, microbial assimilation, and denitrification process (GERSBERG et al., 1986; KADLEC; KNIGHT, 1996; MITSCH; GOSSELINK, 2000; YANG et al., 2001). A lower N removal rate in unplanted systems treating high N polluted water was not surprising. Several experimental studies on N removal in treatment wetlands confirmed that unplanted treatment had a lower N removal compared with the

planted one for many cases (JUWARKAR et al.; 1995; COLEMAN et al., 2001; YANG et al., 2001; LIN et al., 2002). Plants have several intrinsic properties that make them an important component of the constructed wetland buffer zone, in addition to many indirect beneficial effects on water quality (GUMBRICHT, 1993; BRIX, 1994). Planted wetland showed improved effluent quality through overall removal of N from domestic wastewater rather than in unplanted wetlands. In most studies that have included unplanted control, wetlands have showed a marked decline in the N removal (VYMAZAL, 1999; COLEMAN et al., 2001; MERLIN et al., 2002), as well as in this work (Figure 3). Wetland plants provide a source of C, directly from roots or from decaying shoots what has been shown to improve N removal efficiency wastewater (GERSBERG et al., 1986). Tanner (2001) reviewed several studies in which planted and unplanted wetlands were compared; he concluded that macrophytes not only marginally increase the rate of elimination of organic matter but clearly increase the rate of removal of ammonium. Plant uptake is the major removal mechanism in constructed wetlands with free-floating macrophytes. The potential of emergent plants is quite low especially in constructed wetlands for the treatment of municipal or domestic sewage (VYMAZAL, 2007).

Phosphorus

Similarly to the nitrogen removal, the phosphate removal capacidade in CWs vegetated were also lower than unplanted systems in this study. In the absence of plants, the gravel substrate provided significant wastewater treatment (COLEMAN et al, 2001), although most studies report improved nutrient removal where plants are present (BREEN 1990; MANN, 1990; TANNER et al, 1995a; HUNTER et al, 2001).

Phosphorus in wetlands occurs as phosphate in organic and inorganic compounds. Free orthophosphate is the only form of phosphorus believed to be utilized directly by algae and macrophytes, but the wetlands provide an environment for the interconversion of all forms of phosphorus (VYMAZAL, 2007). Some of the phosphorus transformations in wetlands are: peat/soil accretion, adsorption/desorption, precipitation/dissolution, plant/microbial uptake and others (VYMAZAL, 2001). The phosphorus is taken up by plants roots, absorption through leaves and shoots is restricted to submerged species but this amount is usually very low (VYMAZAL, 2007). Most P is believed to be stored in the media bed, rather than in the plant. Studies in the larger scale *P. australis,* gravel P sorption increased and accounted for 30% P removal (HEADLEY et al., 2001). In this study, *Phragmites* sp. also was more efficient in the phosphate removal in the SSF regimen. Adsorption and precipitation of phosphorus is effective in systems where wastewater gets in contact with filtration substrate. It means that constructed wetlands with sub-surface flow have the major potential for phosphorus removal via these mechanisms (VYMAZAL, 2007).

Peat/soil accretion is the major long-term phosphorus sink in wetlands but it could be effective only in treatment wetlands with high production of biomass and water overlying the sediment as it is the case of free water surface constructed wetlands with emergent vegetation. The capacity of a reed bed to remove P may be dependent on the contents of minerals in the substrate (ARIAS et al., 2001). Thereby, phosphorus is removed primarily by ligand exchange reactions, where phosphate displaces water or hydroxyls from the surface of Fe and Al hydrous oxides. However, media used for HSF wetlands (e.g. pea gravel, crushed stones) usually do not contain great quantities of Fe, Al or Ca and therefore, removal of phosphorus limited by sorption capacity of the filtration materials used (VYMAZAL, 2005). It should be remembered that the P-removal in full-scale systems occurs not only by P-sorption to the

medium, but also through incorporation into organisms (biofilms and plants) and the subsequent accumulation of organic matter in the systems (ARIAS et al., 2001).

Plant uptake (and assimilation)

The contribution of plants in removing nutrients varies with the nature the effluent and the age of the wetland (HUETT et al., 2005). In temperate climates, macrophyte uptake is a spring-summer phenomenon. Plants such as *Typha* spp. or *Phragmites australis* in northern climates have an obvious annual cycle of aboveground biomass, and the vegetation nutrient concentration tend to be highest early in the growing season, decreasing as the plant mature and senescence (VYMAZAL, 2007). In addition, plant uptake removal mechanisms are limited by temperate and colder regions because regimes do not allow the harvesting of macrophytes, and especially *P. australis*, during the peak nutrient standing stock in the late summer (VYMAZAL, 2005). In this study there was not harvesting, but this phenomenon could explain the seasonal variation observed in the reduction of nitrogen and phosphate removal, mainly in the summer/2008 (Figure 2 and 3).

The seasonal dynamics of the removal of nutrients (ammonium and phosphate) was more evident in the CWs with *Typha* sp., where the efficiency removal was decreasing since summer/2007 to summer/2008, with exception of the removal of phosphate in the FFP (CW1), which demonstrates better removal in the summers than in the winter. *Phragmites* sp. presents a seasonal variation with better nutrients removal in the summers. Similarly, results were obtained by Felberová et al. (2001) in the nitrogen removal in a CW planted *P. australis*. It has been reported in the literature (JENSSEN et al., 1993; VYMAZAL 1999) that there is no significant difference between summer and winter. Contrary to that, Brix and Schierup

(1989) and Mander and Mauring (1979) found smaller removal of nitrogen in winter that could be explained by low temperature, as in this study. However, it is still uncertain, whether the low winter capacidades are due to cold temperatures alone or to the combined effects with increased hydraulics loadings, because several other studies have not shown significant treatment effects between winter and summer (BROWN; REED, 1994; NERALLA et al., 2000).

Vymazal (2001) reported that it has also been found that biofilms attached to the rizhomes of *P. australis* support higher potential rates of nitrogen transformations per unit surface area than those attached to gravel. Otherwise, plants of *P. australis* in a gravel-based subsurface flow wetland were required to achieve efficient N and P removal from plant nursery runoff (HUETT et al., 2005) and the capacidade similar obtained in the studies made in a larger scale with the same plant by Headley et al. (2001).

One aspect that has been controversial is the role of vegetation and the effects of different plant species (KARANTHANASIS et al., 2003). Species of choice are certainly to vary with the design and purpose of the wetland, and with the inflowing water quality (IAMCHATURAPATR et al, 2007). The potential of using *Phragmites australis* and *Typha latifolia* in CWs is found in literature in several studies dealing with domestic and industrial wastewater (JUWARKAR et al., 1994; KADLEC; KNIGHT, 1996; VYMAZAL, 2005; VYMAZAL; KRÖPFELOVÁ, 2005). In this study, *Typha* sp. presented a better efficiency in the nutrients removal than *Phragmites* sp., but in some comparisons the opposite occured. Juwarkar et al. (1994) when comparing various plants found out that *Phragmites australis* was slightly better than *Typha latifolia* and in another study *Phragmites carca* was more efficient in N removal compared to *Typha latifolia*. Gersberg et al. (1986) observed that the bulrushes (*Scirpus validus*) and reeds (*Phragmites communis*) proved to be superior at

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removing ammonia, both with average effluent levels significantly below than that for the cattail bed (*Typha latifolia*). They suggested that the high-ammonia-N (and total-N) removal efficiencies shown by the bulrush and common reed beds are attributed to the ability of these plants to translocate O_2 from the shoots to the roots. According to Coleman et al. (2001), the improvements in effluent quality are probably due both to direct nutrient uptake by the plants for growth, and the actions of aerobic microbes harbored in the rizosphere. Their results demonstrate that while *Typha latifolia* was clearly superior in facilitating the treatment processes and was also the stronger competitor, its aggressive nature and aesthetic drawbacks have been cited as reasons to avoid its use in these systems. Calheiros et al. (2007), in a horizontal subsurface CW, evaluated the treatment capacidade a tannery wastewater, tested several species (*Canna indica*, *Typha latifolia*, *Phragmites australis*, *Stenotaphrum secundatum* and *Iris pseudacorus*), but no significant differences were observed in capacidade between units. Despite that, *Typha* sp. and *Phragmites* were the plant species better adapted to tannery wastewater in terms of survival and propagation.

Horizontal flow with emergent plants (FWS and HSSF)

The most widely used concept of constructed wetlands in Europe is the one with horizontal sub-surface flow (SSF or HSSF wetlands), but the treatment systems with emergent macrophytes can be constructed with free water surface (FWS or SF wetlands) (VYMAZAL, 2007). In both designs, during the passage of wastewater through the reed bed (*Phragmites australis,* the specie more frequently used in the SSF), the wastewater treatment wetlands typically have aerated zones; in the FWS, it is especially near the water surface because of the atmospheric diffusion, besides the anoxic and anaerobic zones (VYMAZAL

2005). Tanner et al. (1995b) claim that the role of the substrate and rhizosphere in surface flow systems is quite negligible compared with the subsurface ones, where long residence times allow extensive interaction with the wastewater. While resilient, slow growing species with low seasonal biomass turnover, and high root-zone aeration capacity may be suitable for surface flow systems, high productivity species, tolerant to high levels of pollutants and hypertrophic waterlogged conditions may be functionally superior in subsurface flow systems. The major reason is that FWS CWs have very soil processes limited and sub-surface CWs lack processes in the free water zone. In fact, the magnitude of processes which ultimately remove total nitrogen from the systems is usually low, and therefore, removal of TN is commonly low in single-stage constructed wetlands (VYMAZAL, 2007).

In this study, the removal efficiency varied between 40 and 60% based in average values (Table 4). Likewise, Vymazal (2007) obtained similar results similarly in systems akin varying between 40 and 50% in the removal of total nitrogen, and 40 a 60% in the removal of ammonium in various studied types of CWs and inflow loading.

Table 4. Removal of ammonium (NH_4-N) and phosphate (PO_4-P) in various types of constructed wetlands (mean values).

CW type	Inflow 14.6 (mg NH_4-N L^{-1}) 2.1 (mg PO_4-P L^{-1})	Outflow NH_4-N (mg L^{-1})	Outflow PO_4-P (mg L^{-1})	Efficiency (%) NH_4-N	Efficiency (%) PO_4-P
Concentrations					
FWS	CW2 – *Typha* sp.	7.5	1.6	48.6	23.8
FFP	CW1 - *Typha* sp.	5.6	1.3	61.6	38.1
	CW5 – *Phragmites* sp.	8.7	2.1	40.4	0.0
FWSS	CW3 - *Typha* sp.	6.2	0.8	57.5	61.9
SSF	CW7 - *Phragmites* sp.	6.7	0.1	54.1	95.2
	Inflow 0.73 (g NH_4-N m^{-2} dia^{-1}) 0.11 (g PO_4-P m^{-2} dia^{-1})	Loading NH_4-N (g m^{-2} dia^{-1})	Loading PO_4-P (g m^{-2} dia^{-1})	Removed Load NH_4-N	Removed Load PO_4-N
FWS	CW2 – *Typha* sp.	0.46	0.05	0.27	0.06
FFP	CW1 - *Typha* sp.	0.56	0.06	0.17	0.05
	CW5 - *Phragmites* sp.	0.60	0.04	0.13	0.07
FWSS	CW3 - *Typha* sp.	0.48	0.07	0.25	0.04
SSF	CW7 - *Phragmites* sp.	0.51	0.10	0.22	0.01

The constructed wetlands with FFP and FWSS were more efficient in the removal of nutrients and loading removal of nitrogen both planted with *Typha* sp.*,* and the CWs with FWSS and SSF planted with *Phragmites* sp. and *Typha* sp. to phosphate removal had better efficiency, respectively (Table 4). The CWs with FWS and FWSS (both planted with *Typha* sp.) had loading removal to nitrogen more elevated, differently from loading removal to

phosphate where the better efficiency were in the FFP planted with *Phragmites* sp.*,* FFP and FWS planted with *Typha* sp. (Table 4). Vymazal (2007) also found a slightly higher removal for FFP CWs, but in that case, it is a result of a multiple harvesting.

SF wetland systems generally have a lower contaminant removal efficiency compared with SSF systems (LEE et al., 2009). Vymazal et al. (1998) related that subsurface flow constructed reed beds generally have a greater potential to remove nitrogen rather than phosphorus, because nitrogen can be converted to gas and be emitted to the atmosphere as a consequence of coupled nitrification/ denitrifcation processes. Although, Lin et al. (2008) studied the effects of hydraulic loading rate on capacidade of the FWS and SSF constructed wetlands in nitrate removal, not observing significant difference between the two types of system.

Wetland hydraulic: loading rate, porosity, hydraulic retention time and harvesting of plants

The capacidade of existing CWs has varied widely due to the influences of diverse natural factors and design parameters, such as the type of wastewater, hydraulic retention time, loading rate (USEPA, 2000; TAO et al., 2006). Specifically, in a FWS wetland, the vegetation, settled solids, litter and peat occupied a portion of the water column, thereby reducing the space available for water. The porosity of a wetland (ε), or void fraction, is the fraction of the total volume available through which water can flow. The overall effects of decreasing porosity are to reduce the wetland volume available for water, which reduces the retention time of water within the wetland (USEPA, 2000), that was exactly what occurred with CW1 in relation to CW5. The best performance of this wetland could be related to more

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plants at CW1, consequently showing a more elevated root volume in relation to CW5, those might to elevate root zone aeration capacity. Brix (1997) reported that wetlands plants rooted provide additional attachment surfaces for microbes, and assimilate nutrients. They may also affect treatment processes by modifying environmental conditions in the bed through root-zone release of oxygen, and promoting nitrification and other aerobic processes (TANNER et al., 1998).

The same aspects evaluated previously, but now in the wetlands with gravel comparing FWS and FWSS (CW2 and 3, respectively) revealed that FWSS was more efficient and showed a high porosity in the bottom and a more elevated HRT. Brix (1993) reported that in gravel-plant constructed wetland systems, since both processes of nitrification-denitrification and plant uptake are the main removal mechanisms for N due to their high porosity, the removal efficiencies of N are supposed to be higher than those in soils. Wu et al. (2008) working with mangrove sub-surface flow microcosms, as well in these results, had better ammonia removal the HRT of 10 days than that of 5 days. Similarly, White (1995) showed that the ammonia removal efficiency by the wetland system with the 10-day HRT was two times that with 2.5-days HRT. Ammonia N removal was generally low in the two SSF CWs and was clearly affected by the HRT, besides having the highest removal rates with 6d HRT when the effluent concentrations were also higher (CASELLES-OSORIO; GARCÍA, 2006). Reddy and D'Angelo (1997) reported that ammonia removal might in this study have been more affected by HRT because processes such as nitrification have slower rates. Wieβner et al. (2005) related that owing to the usually slow flow rates and the resulting long hydraulic retention times, the spatial gradients favor oxidative processes in layers near the root surface and mainly reductive processes farther away from the roots.

Similar results to those previously mentioned were observed in CW3 relative to CW7, both with gravel but now comparing FWSS and SSF and comparing *Typha* sp. and *Phragmites* sp., where FWSS was more efficient with bigger porosity and high HRT. These results were observed to volumetric and superficial loading removal do nitrogen, except in the winter/2008. Yang et al. (2001) related that for gravel-bed systems, compared to soil-bed systems, a higher porosity would allow more oxygen transfer to the substratum and more biomass would accumulate inside the substratum, both of which would be helpful for nitrification (YANG et al., 2001).

The biggest loading removal to phosphate was of CW7 with SSF and gravel, and also was bigger the loading removal to nitrogen in the winter/2008; in this case the systems presents lesser porosity and HRT. Contradictory results were also observed by García et al. (2004), in akin systems, operated with SSF and *Phragmites* sp., where the better capacidade was obtained with a lower hydraulic retention time (approximately from 2.5 to 5.5 days). Huett et al. (2005) related that plant uptake was the dominant removal mechanism reducing TP with 3.5-day reaction time. According to, in our results it was possible to observe an increase percentage of phosphorus removal in *Phragmites* sp. in relation to *Typha* sp. (Table 4). It should be remembered that the P-removal in full- scale systems occurs not only by P-sorption to the medium, but also through incorporation into organisms (bioflms and plants) and the subsequent accumulation of organic matter in the systems. Therefore, even when the P-removal capacity of the medium is completely exhausted, some P-removal in the system will still occur (ARIAS et al., 2001). Add to this, Headley et al. (2001) also achieved similar capacidade in a larger scale *P. australis* study where P removal through gravel fixation and detritus accumulation and sedimentation became the significant nutrient removal processes.

Other interesting aspect is that the gravel occupies a considerable proportion of the bed volume in these systems, and the growth of biofilms and plant root systems within these void spaces further reduce their effective interstitial volume. As the gravel matrix gradually clogs, both its hydraulic conductivity and the effective residence time of wastewater flowing through it is reduced, affecting flow pathways (e.g. promoting surface flow) and ultimately the treatment capacidade of wetland (TANNER et al., 1998). Preliminary results of Tanner and Sukias (1995), in gravel-bed wetlands treating farm dairy wastewaters, indicated that organic matter accumulation could be substantial. Tanner et al. (1998) still related that, over a long-term, this accumulation may have significant effects on the hydraulic retention time and the capacity of the wetlands to retain nutrients. In this work, the decline in P removal efficiency over time by gravel-based wetlands was observed.

9.5. CONCLUSIONS

The CWs presented broad variation the data of pH, DO concentration and redox potential, and these differences were also seasonal. The seasonal variation occurred including differences between the summer 2007 and 2008. This sustains, in fact, that the CWs can be considerably dynamic systems. The ammonium and phosphate removal rates were presented within the ranges reported by literature, and the planted CWS with macrophytes were more efficient in relation to unplanted systems. In general, *Phragmites* sp. was more efficient in the nutrient (ammonium and phosphate) removal, but this aspect ranged depending on flow of regimen and the season. The constructed wetlands with FFP and FWSS were more efficient in the removal of nutrients and loading removal of nitrogen both in the systems planted with *Typha* sp*.,* and the CWs with FWSS and SSF planted with *Phragmites* sp. and *Typha* sp. (to

phosphate removal had better efficiency). The seasonal dynamic of the removal of nutrient was more evident in the CWs planted with *Typha* sp. The FWSS presented better capacidade in the nutrient removal and the CWs are characterized by this flow showed increase in the porosity and more elevated HRT.

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CAPÍTULO 10. CONSIDERAÇOES FINAIS

Considerando os diferenets experimentos desenvolvidos, conclui-se que:

a) Os estudos dos mecanismos de detoxificação e sensibilidade ao cromo em *Pseudokirchneriella subcapitata* confirmam a utilização desta espécie como bioindicador de contaminação ambiental causada por este metal.

b) A relação entre a bioacumulação de cromo em *P. subcapitata* e a variação no biovolume desta alga sugere uma estratégia da espécie para suportar elevadas concentrações de cromo. A redução do biovolume poderia representar um aumento na superfície específica das células que aumentam os sítios de ligações do metal, ampliando assim a capacidade de bioacumulação. Investigações mais detalhadas poderiam confirmar esta estratégia, reforçando seu uso na bioindicação e na biorremediação.

c) *Pistia stratiotes* demonstra um padrão diferencial de bioacumulação entre as raízes e a parte aérea. O acúmulo dos metais cromo e cobre foram mais intensos na raiz em relação à parte aérea, corroborando com os dados da literatura, a qual relata que é pobre a translocação entre estes órgãos vegetais.

d) Neste sentido, foi possível observar que a peroxidação de lipídios e a indução das enzimas em defesa ao estresse oxidativo causado pelo cromo e pelo cobre em *P. stratiotes*, apresentaram respostas mais intensas nas raízes. A Catalase (CAT) representa o principal sistema de defesa das células vegetais, no sentido de evitar o acúmulo de peróxido de hidrogênio, substância produzida pela ação de neutralização das espécies reativas de oxigênio (ERO). Os resultados revelaram que a atividade desta enzima, foi mais intensa nas raízes de *P. stratiotes* submetidas ao estresse causado por cromo e cobre. No caso da glutationa redutase (GR), a atividade também foi mais intensa na raiz em relação à parte aérea para as macrófitas cujo estresse foi induzido por cobre.

e) O conteúdo de clorofila em plantas é considerado como um dos parâmetros mais sensíveis sob condição de estresse causado por metais. Inicialmente houve aumento no teor de clorofila em *P. stratiotes* submetido ao estresse induzido por cromo e cobre, o qual foi decrescendo no decorrer do tempo, em concentrações mais elevadas dos referidos metais. Resultados estes, que contrariam os da literatura atual. O teor de clorofila *a* foi mais afetado do que o teor de clorofila *b* em ambos os tipos de estresse. Lembrando que a clorofila *a* é o pigmento mais importante dentro dos fotossistemas e seu decréscimo pode afetar consideravelmente a fotossíntese.

f) A emissão de fluorescência pelas moléculas de clorofila representa a dissipação da energia luminosa que não será utilizada nas reações fotoquímicas, proporcionando medidas da eficiência fotoquímica do fotossistema II (PSII). Vários coeficientes podem ser utilizados para calcular a dissipaçao de energia pelos fotossistemas e neste caso, o rendimento fotossintético do PSII (Yeild ou ΦPSII) e o Índice de Vitalidade (R_{fd}) foram os mais sensíveis no estresse causado pelo cromo em *P. stratiotes*.

g) Apesar dos resultados pouco expressivos, o uso do método do Pulso de Amplitude Modulada (PAM fluorométrico) da fluorescência da clorofila na ecotoxicologia aquática pode ser considerado válido por várias razões:

(1) por se tratar de um método rápido, sensível, prático e não destrutivo que permite avaliações *in situ* e em bioensaios, as quais ampliam consideravelmente a relevância fisiológica dos resultados observados;

(2) os metais geralmente afetam processos fisiológicos relacionados à fotossíntese, desta forma a detecção de inibição fotossintética representa um aspecto seguro e confiável do potencial fitotóxico de ambientes contaminados por metais;

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(3) a ampliação de seu uso ainda é restrita devido ao volume reduzido de informações com relação à sensibilidade de diferentes espécies de algas e plantas às respostas dos parâmetros relativos à emissão de fluorescência a uma ampla faixa de poluentes.

h) De uma maneira geral, pode-se dizer que *P. stratiotes* desenvolve mecanismos de detoxificaçao e defesa, os quais permitem a espécie tolerar condições de estresse causado por concentrações nominais de cromo ($1,0 - 6,0$ mg Cr L^{-1}) e de cobre ($2,0$ a $10,0$ µg Cu L^{-1}) permitindo indicá-la como bioindicadora e biorremediadora destes metais.

i) A bioacumulaçao em *P. stratiotes* ocorre no sistema radicular, porém ainda existem lacunas na compreensão dos mecanismos de detoxificaçao; se ocorrem por adsorção dos metais, quelaçao, troca iônica, ou por precipitação radicular.

j) Os resultados obtidos durante o desenvolvimento desta pesquisa e a experiência adquirida com o uso do Delineamento Composto Central (DCC) e da Metodologia de Superfície de Resposta (MSR) permitem recomendar seu uso na ecotoxicologia aquática por várias razões:

- esta metodologia permite avaliar vários fatores simultaneamente, bem como avaliar as interações entre estes fatores;

- reduz o número de experimentos, sem perdas na confiabilidade dos dados e com conseqüente redução no volume de resíduos gerados ao final de cada experimento;

- permite gerar modelos preditivos de toxicidade e a simulação dos referidos modelos amplia a compreensão dos mecanismos de toxicidade.

k) As macrófitas representam um grupo de plantas que têm sido amplamente utilizadas na área de biorremediação (como bioacumuladora de metais) e na remoção de nutrientes em

wetlands contruídos. Os wetlands artificiais representam sistemas extremamente dinâmicos, sítio de ocorrência de vários processos físicos, químicos e biológicos, aspectos observados através dos parâmetros avaliados e da variação sazonal observada durante o período experimental.

l) *Typha* sp. e *Phragmites* sp. estão entre as plantas mais comumente utilizadas nos wetlands construídos e sua presença amplia as condições de filtração do sistema, assim como a área de superfície de interação das raízes com microrganismos e dos nutrientes com as plantas, aumentado a capacidade de remoção dos nutrientes. Isso, sem levar em consideração, inúmeros outros aspectos que revelam sua importância nos wetlands. Os resultados observados revelaram que os wetlands plantados apresentaram maior eficiência na remoção de nutrientes (amônia e fosfato) quando comparado aos wetlands não plantados.

m) Por outro lado, a eficiência da espécie na remoção dos nutrientes depende do regime de fluxo e das condições hidráulicas aplicadas no sistema. Nos wetlands sob cultivo hidropônico e com fluxo subsuperficial com a superfície livre de água, plantados com ambas as macrófitas (*Typha* sp. e *Phragmites* sp.), houve boa eficiência de remoção de nutrientes. A influência da variação sazonal na dinâmica de remoção de nutrientes foi mais evidente nos wetlands plantados com *Typha* sp.

n) Os sistemas com fluxo subsuperficial com a superfície livre de água foram os wetlands que desempenharam melhor *capacidade* na remoção de nutrientes, porque apresentaram maior porosidade e maior tempo de retenção hidráulica.

A poluição dos corpos d´água tem sido um problema constante na atualidade, e estudos como estes oferecem subsídios para propostas futuras de preservação e recuperação ambiental. O uso do DCC e da MSR oferecem condições de simulação de dados e modelos de

predição de toxicidade causada por metais, visando ainda à redução na geração de resíduos experimentais. As análises enzimáticas e de fluorescência da clorofila fornecem metodologias mais sensíveis na avaliação da contaminação ambiental causada por metais. Outro aspecto importante a ser considerado, é que este estudo amplia os conhecimentos sobre as macrófitas, e sua aplicação na descontaminação ambiental em corpos d´àgua e em sistemas de depuração de águas residuárias.

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