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IAGO TELES DOMINGUEZ CABANELAS

CULTIVO DE MICROALGAS EM EFLUENTES DOMÉSTICOS:
AVANÇO TECNOLÓGICO PARA PRODUÇÃO DE BIOCOMBUSTÍVEIS

Salvador
2012

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Dissertação apresentada ao Programa de Pós-Graduação em Biotecnologia do Instituto de Ciências da Saúde da Universidade Federal da Bahia como requisito parcial para obtenção do titulo de mestre em biotecnologia.

Orientador: Dr. Paulo Fernando de Almeida
Co-orientadora: Dr[a] Iracema Andrade Nascimento

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A

Carminha e Cabanelas, amados pais
Yolanda, querida e saudosa avó

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RESUMO

No atual cenário energético mundial é notória a insustentabilidade do uso continuado de combustíveis fósseis, devido à projetada diminuição do suprimento de petróleo, e à contribuição destes combustíveis para o acúmulo de dióxido de carbono na atmosfera. Como, da demanda bruta por energia, no mínimo 60% é absorvida pelo setor de transportes, torna-se lógico propor a substituição dos combustíveis fósseis pelos biocombustíveis como alternativa para redução de emissões poluidoras. As microalgas são uma possibilidade versátil uma vez que podem dar origem a, além de biodiesel (óleo), etanol (fermentação de amido), bioquerosene (hidrocarbonetos líquidos), bioplásticos (biopolímeros), biohidrogênio, biogás (metano) e outros derivados químicos. O uso de resíduos domésticos vem se destacando como opções economicamente viável e efetivamente eco-compatível para o cultivo de microalgas. A presente proposta teve o objetivo de avaliar o potencial de efluentes domésticos urbanos como fonte alternativa de nutrientes para o cultivo de microalgas. O desenvolvimento do presente trabalho foi dividido em dois artigos científicos elaborados com os dados experimentais. Os artigos mostram (i) o efeito de diferentes águas residuárias sobre a microalga *Chlorella vulgaris* e (ii) os efeito da adição de diferentes concentrações de glicerina sobre o metabolismo das espécies *Chlorella vulgaris* e *Botryococcus terribilis* cultivadas em água residuária. A microalga *Chorella vulgaris* apresentou crescimento satisfatório em diferentes efluentes domésticos tratados, alcançando produtividades entre 50 e 150 mg L^{-1} d^{-1}. Em todas as amostras foi observada a remoção de nutrientes. A suplementação de CO_2 é uma importante variável para a produção de biomassa e eficiente remoção de nutrientes. *Chlorella vulgaris* foi capaz de fixar como biomassa entre 56 e 242 mg por litro por dia de CO_2. Modificações artificiais da razão N/P não aumentou a produção de biomassa nem a taxa de remoção de nutrientes, para os cultivos com efluente da secagem dos lodos. Para os experimentos com adição de glicerina não foi possível estabelecer correlação linear entre as concentrações de glicerina e a produção de biomassa. Contudo, foi observado aumento significativo de biomassa com 25 e 50 mM de glicerina. Tais resultados indicam a possibilidade de aplicar a glicerina como fonte de carbono orgânico para a produção de biomassa microalgal. Também foi possível observar altos valores de remoção de nutrientes, acima de 70% para DQO, nitrogênio e fósforo. Contudo, as produtividades lipídicas, embora não correlacionadas com a disponibilidade de glicerina foram reduzidas com a disponibilidade deste nutriente. Tais resultados apontam a glicerina como indicada para produção de biomassa de microalgas, porém não visando altas produtividades lipídicas.

Palavras-chave: Microalgas; efluentes domésticos; biocombustíveis.

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ABSTRACT

In the current world energy scenario is notorious the unsustainability of the continuous use of fossil fuels, due to projected decrease in petroleum supply, and the contribution of these fuels to the accumulation of carbon dioxide in the atmosphere. Since, from the gross demand for energy, at least 60% is absorbed by the transportation sector, it is logical to propose the substitution of fossil fuels, by biofuels, as an alternative to reduce polluting emissions. Microalgae are a versatile possibility since they may produce, besides biodiesel (oil), ethanol (fermentation of starch) biokerosene (liquid hydrocarbons), bioplastics (biopolymers) biohydrogen, biogas (methane) and other chemical derivatives. The use of domestic wastewater has emerged as viable option for effectively and eco-compatible cultivation of microalgae. This proposal aimed to evaluate the potential of urban wastewater as an alternative source of nutrients for the cultivation of microalgae. The development of this work was divided in two papers prepared with the experimental data. The research articles report (i) the effect of different effluents on the microalgae *Chlorella vulgaris* and (ii) the effect of adding different concentrations of glycerol on the metabolism of the species *Chlorella vulgaris* and *Botryococcus terribilis* cultivated in wastewater. The microalgae Chorella *vulgaris* showed satisfactory growth in different treated effluents, reaching yields between 50 and 150 mg L-1 d-1. All samples showed efficient nutrient removal. The CO2 supplementation is an important variable for the production of biomass and efficient removal of nutrients. *Chlorella vulgaris* was able to fix in biomass from 56 to 242 mg per liter per day of CO_2. Artificial modifications to N/P ratios did not increase biomass production or the rate of removal of nutrients for cultures with effluent from the sludge drying. In the experiments with glycerol addition, it was not possible to establish linear correlation between the concentrations of glycerol and biomass production. However, we observed a significant increase in biomass at 25 and 50 mM glycerol. Such results suggest the possibility of applying glycerol as organic carbon source for producing microalgal biomass. It was also possible to observe high values of nutrient removal, above 70% for COD, nitrogen and phosphorus. However, the lipid yields, even though not correlated with the availability of glycerol, were reduced with the availability of this nutrient. These results indicate glycerol as suitable for biomass production of microalgae, but not aiming high lipid yields.

Keywords: Microalgae; domestic wastewater; biofuels.

SUMÁRIO

1. INTRODUÇÃO

No atual cenário energético mundial é notória a insustentabilidade do uso continuado de combustíveis fósseis, devido à projetada diminuição do suprimento de petróleo, e ainda à contribuição destes combustíveis para o acúmulo de dióxido de carbono na atmosfera, e conseqüente aumento do efeito estufa (CHISTI, 2007). O Relatório do Painel Intergovernamental sobre Mudanças Climáticas (IPCC AR4 Shynthesis Report) é claro em apontar os níveis atuais de CO_2 atmosférico (acima de 380 ppm), como tendo atingido o patamar de perigo. Por outro lado, o Relatório Stern (The Economics of Climate Change, 2006) mostra que o aquecimento global em 2 a 3^{o} C pode levar a perdas permanentes anuais de até 3% do PIB mundial (US$ 1,32 trilhão), o que faz do desenvolvimento de tecnologias CO_2-neutras um dos mais urgentes desafios para a humanidade neste século.

Adicionalmente, o aumento da população mundial, que já atingiu a marca de 7 bilhões de habitantes, tem determinado a crescente demanda por recursos. A demanda de energia, em termos mundiais, deverá crescer em 57% sobre os valores atuais, até 2025 (IEA, 2007). A necessidade de segurança no suprimento de energia e de produzi-la e utilizá-la de uma forma ambientalmente sustentável tem direcionado um deslocamento nas matrizes energéticas de cada país, visando a substituição das fontes fósseis por renováveis (OECD, 2009). Estas conclusões estão levando as principais lideranças mundiais a estabelecerem metas para a redução de emissões de CO_2 entre 10-20% até 2020, Entretanto, mesmo projeções otimistas não mostram possibilidade de estabilização para os valores de CO_2 atmosférico. Desta forma, estima-se que apenas uma redução de 60% das emissões até 2020 pode gerar resultados eficazes, o que não pode ser alcançado com o atual nível de desenvolvimento em termos de tecnologias CO_2 neutras (OECD, 2009).

Atualmente já se encontram disponíveis alternativas para o setor de fornecimento de energia elétrica (que representa em média 30% da demanda energética global), no sentido de redução das emissões de CO_2 (energia nuclear, solar, eólica, hidrelétrica, geotérmica). Contudo, o setor de biocombustíveis ainda carece de desenvolvimento tecnológico, uma vez que segue baseado em tecnologias de primeira geração. Tais tecnologias, embora estabelecidas em larga escala, ainda apresentam alto custo e não são competitivas em nível de mercado, ao ponto de permitir a substituição significativa dos combustíveis fósseis. Entre elas estão, a transformação do óleo vegetal e de gorduras animais em biodiesel,

essencialmente realizadas por transesterificação e ainda a transformação de carboidratos em etanol.

Como, da demanda bruta por energia, no mínimo 60% é absorvida pelo setor de transportes (SCHENK, 2008), torna-se lógico propor a substituição dos combustíveis fósseis pelos biocombustíveis como alternativa para redução de emissões poluidoras. Os biocombustíveis apresentam vantagens por serem biodegradáveis e reduzirem as principais emissões presentes nos gases de exaustão, com exceção dos óxidos de nitrogênio (no caso do biodiesel). Tais vantagens podem ser ampliadas com o aproveitamento da grande biodiversidade que o Brasil apresenta, pois as muitas espécies, capazes de produzir óleo para o biodiesel, crescem bem em seu solo agrícola (TEIXEIRA *et al*., 2006), além do que a cana de açúcar gera o etanol com a maior eficiência entre as matérias–primas deste biocombustível. Todavia, o custo ambiental da expansão agrícola necessária à produção de biocombustíveis de primeira geração é alto, devido à necessidade de ampliação das monoculturas (e das alterações no uso de solos, liberando CO_2, por exemplo), ao uso intensivo de agrotóxicos e fertilizantes que podem determinar, por lixiviação, poluição de corpos de água, além da redução deste recurso hídrico, redução de habitats e perda de biodiversidade (SHUCHARDT *et al.*, 1998). Estudos recentes (ZAH *et al*.,2007; ALTIERI, 2009) apresentam resultados de análise de ciclo de vida (ACV) de biocombustíveis de primeira geração que os posicionam, em alguns casos, acima dos combustíveis fósseis como causadores de impactos ambientais. Para substituir os combustíveis fosseis fazem-se necessárias alternativas efetivamente eco-compatíveis e a custo competitivo, o que pode ser alcançado com uso de microalgas como matéria-prima para biocombustíveis.

As microalgas representam um grupo de microorganismos extremamente diverso, embora altamente especializado e de ampla distribuição biogeográfica. Muitas destas espécies já foram isoladas e encontram-se mantidas em coleções em diversas instituições. As microalgas são uma possibilidade versátil uma vez que podem dar origem a, além de biodiesel (óleo), etanol (fermentação de amido), bioquerosene (hidrocarbonetos líquidos), bioplásticos (biopolímeros), biohidrogênio, biogás (metano) e outros derivados químicos (CHISTI, 2007).

O aspecto qualitativo da biomassa microalgal tem obtido destaque nos últimos anos com o surgimento do conceito de biorrefinaria, que posiciona as microalgas como uma fonte

diversa de matérias-primas e com aplicação em variados setores produtivos. Tal modelo de aplicação marca um avanço na lógica de mercado e evidencia o espaço que a bioenergia vem ganhando em escala mundial e o interesse global em se alcançar a sustentabilidade ambiental com eficiência energética.

As microalgas além de, comparativamente com outras oleaginosas, exigirem um menor gasto de água (por possibilitar o reuso) e produzirem maior quantidade de biomassa (cerca de 1 kg/m³de cultura/dia), geram mais óleo vegetal (de 35 a 70% da biomassa a depender da espécie e das condições de cultivo**)** (METZGER;LARGEAU, 2005; WALTER *et al*., 2005; SPOLAORE *et al*., 2006). As microalgas são fixadoras de CO2, registrando-se que, para cada quilo de biomassa algal produzida, são consumidos 1.5 a 2 Kg de CO2, o que representa entre 10 a 20 vezes mais que o absorvido pelas culturas oleaginosas (BROWN; ZEILER, 1993). Reproduzem-se rapidamente; durante a fase exponencial de crescimento, o tempo de duplicação da biomassa é de praticamente 3.5 h (SPOLAORE *et al*., 2006). Os teores de ácidos graxos e triglicerídios (TG) dependem das espécies em cultivo e das condições da cultura, devendo estes fatores ser considerados para o direcionamento ao produto-foco.

Apesar de tantas vantagens e de já serem exploradas comercialmente, as microalgas ainda não estão inseridas na matriz energética mundial. Por apresentaram custos elevados, a produção comercial é focada em produtos de maior valor agregado (suplementos alimentares, farmacêuticos, corantes e alguns compostos químicos finos). Para a aplicação da biomassa algal à indústria energética, uma série de entraves e gargalos tecnológicos devem ser superados. Os entraves iniciais para a produção de biomassa microalgal envolvem a bioprospecção (identificação de cepas resilientes, de rápido crescimento e altamente produtivas em lipídios neutros) e a busca de uso de resíduos como fontes de nutrientes para os cultivos. A otimização dos parâmetros e sistemas de cultivo são uma outra frente de trabalho, que incluí ainda desenvolvimento de linhagens celulares transgênicas. Outras dificuldades referem-se ao custo energético dos processos de colheita e extração do óleo a partir da biomassa.

O uso de águas residuárias domésticas e industriais vem se destacando como opções economicamente viáveis e efetivamente eco-compatíveis (RAWAT *et al*., 2011; LARSDOTTER, 2006; PARK *et al*., 2011) para o cultivo de microalgas. Águas residuárias constituem um potencial meio de cultivo para microalgas devido à riqueza em compostos orgânicos e

inorgânicos e à necessidade de sua remoção (especialmente N e P, vide Tabela 1), uma vez que sua emissão em ecossistemas naturais gera riscos tanto à saúde humana quanto ambientais (causam eutrofização e depleção de oxigênio).

Tabela 1: Composição típica de águas residuárias não tratadas. Adaptado de Rawat e colaboradores (2011)

Composição		**Concentração**		
Contaminantes	**Unidade**	**Fraca**	**Média**	**Forte**
Sólidos totais	mg L^{-1}	350	720	1200
Sólidos dissolvidos totais	mg L^{-1}	250	500	850
Fixos	mg L^{-1}	145	300	525
Voláteis	mg L^{-1}	105	200	325
Sólidos em suspensão	mg L^{-1}	100	220	350
Fixos	mg L^{-1}	20	55	75
Voláteis	mg L^{-1}	80	165	275
Sólidos decantáveis	mg L^{-1}	5	10	20
BOD_5, 20 °C	mg L^{-1}	110	220	400
Carbono orgânico total (COT)	mg L^{-1}	80	160	290
Demanda química de oxigênio (DQO)	mg L^{-1}	250	500	1000
Nitrogenio (total em N)	mg L^{-1}	20	40	85
Orgânico	mg L^{-1}	8	15	35
Amônia (NH_4)	mg L^{-1}	12	25	50
Nitrato (NO_3)	mg L^{-1}	0	0	0
Nitrito (NO_2)	mg L^{-1}	0	0	0
Fósforo (total em P)	mg L^{-1}	4	8	15
Orgânico	mg L^{-1}	1	3	5
Inorgânico	mg L^{-1}	3	5	10
Cloretos	mg L^{-1}	30	50	100
Sulfatos	mg L^{-1}	20	30	50
Alcalinidade (em CaCO3)	mg L^{-1}	50	100	200
Gorduras	mg L^{-1}	50	100	150
Coliformes totais	No/100 mL	10^6-10^7	10^7-10^8	10^7-10^9
Compostos orgânicos voláteis	µg/L	<100	100-400	>400

Tais conseqüências tornam necessário o tratamento destes efluentes visando especialmente a remoção de sua carga química em sistemas de tratamento de efluentes, processos usualmente de alto custo com uso de técnicas tradicionais (DE PAUW;VAERENBERGH, 1983; LARSDOTTER, 2006; RAWAT *et al.*, 2011). Acoplar o cultivo de microalgas com o tratamento de águas residuárias apresenta-se como boa opção para

desenvolvimento tecnológico e comercial uma vez que de um mesmo bioprocesso pode-se atingir de modo mais eficaz e com menor custo dois objetivos: o tratamento de efluentes domésticos e a produção de biomassa microalgal para suprimento da indústria de bioenergia.

2. REVISÃO DE LITERATURA

Descargas de efluentes sem tratamento podem sobrecarregar os sistemas biológicos que, em condições naturais, realizam o processo de depuração e mantém o equilíbrio ambiental. Entre os principais riscos está a eutrofização, causada pelo excesso de nutrientes, que provoca a proliferação do fitoplâncton. Adicionalmente estas descargas podem gerar problemas de saúde pública pela introdução de patógenos e contaminantes químicos no meio natural. Deste modo, o processamento e a destinação devidos de efluentes domésticos e industriais são uma exigência para a sustentabilidade (SYDNEY *et al.*, 2011).

Os tratamentos tradicionais de efluentes domésticos em estações de tratamento de efluentes (ETEs) incluem a remoção de partículas maiores, areias e gorduras (tratamento primário); a redução da demanda biológica de oxigênio (DBO) e de amônia com uso de lodos ativados, filtros e lagoas de estabilização (tratamento secundário) e processos de nitrificação-denitrificação (com uso de bactérias heterotróficas e autotróficas), além de remoção de fósforo (usualmente com precipitação química) e de desinfecção (tratamento terciário). A figura 1 sintetiza os principais métodos de processamento de efluentes domésticos.

Embora sejam eficientes em remover alguns compostos, especialmente derivados de C, N e P, os processos químicos apresentam o inconveniente dos custos de aquisição de reagentes e ainda um aumento do custo ambiental pela possibilidade de contaminação dos corpos d'água, quando os efluentes tratados são lançados no ambiente natural. (CHAN *et al*., 2011). Os processos biológicos se apresentam como os mais eco-compatíveis dentre os disponíveis para ETE. Tais processos utilizam microorganismos para quebrar compostos químicos presentes em efluentes domésticos (RAWAT *et al*., 2011), tornando-os de mais fácil tratamento ou passíveis de vertedura. Processos biológicos são conhecidos há décadas com aplicação ao tratamento de efluentes (PARK *et al*., 2011). Contudo, alguns processos biológicos apresentam requerimentos, operacionais e de insumos, que apresentam custos altos. Contudo, o maior entrave com a sua aplicação está na padronização e controle de estabilidade, uma vez que envolvem sistemas vivos, naturalmente variáveis.

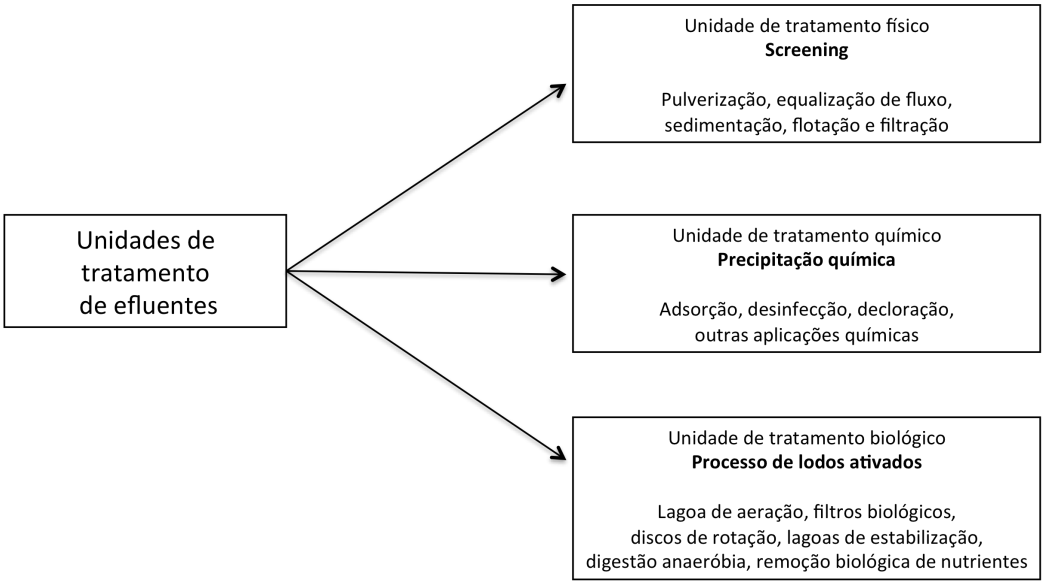

Figura 1: Processos de tratamento de efluentes domésticos comumente utilizados (adaptado de Rawat et al., 2011).

Cultivo de microalgas em águas residuais para diversos fins biotecnológicos já vem sendo praticado há várias décadas com resultados positivos em produtos de alto valor agregado (corantes, suplementos para aqüicultura e derivados químicos) (RICHMOND, 2004). Oswald (1957) foi pioneiro na aplicação de microalgas para fotobiodepuração de efluentes domésticos, demonstrando o potencial destes processos ainda na década de 1940-1950, Foi também o responsável pelo desenvolvimento das primeiras lagoas de estabilização de alta taxa (HPSP), consideradas como precursoras dos fotobioreatores (FBRs) atuais. Benemann (1980) comprovou a eficiência do uso de microalgas para remoção de nutrientes de efluentes domésticos, apresentando uma série de avanços tecnológicos, para o tratamento terciário de efluentes no que tange a otimização da remoção de compostos, essencialmente N e P.

De Pauw (1983) sintetizou os principais avanços tecnológicos na área, envolvendo as duas décadas anteriores. Ressaltou o papel das microalgas em lagoas de estabilização (também chamadas lagoas de oxidação) como indutoras da precipitação do fósforo, redução de amônia e de mal odores através da elevação do pH, conseqüência da liberação de O_2, co-produto da fotossíntese. Indicou as principais variáveis a serem otimizadas: luminosidade, temperatura (ambas marcadamente importantes em países de altas latitudes), área de

superfície para captação solar e o tempo hidráulico de residência dos efluentes nas lagoas, que podem colapsar o sistema por excesso de matéria orgânica. Apesar de bem sucedida, esta tecnologia apresentava como limitação principal a falta de controle da proliferação de biomassa algal, bem como sua contaminação, o que acabou por converter-se em uma das motivações para o desenvolvimento de desenhos de sistemas de cultivos mais eficientes e produtivos, os atuais fotobioreatores.

Após um período de menor avanço tecnológico, entre 1985 a 1995 uma nova abordagem que combina tratamento de efluentes com a produção de energia renovável colocou o assunto em evidência, uma vez que trouxe duas novas possibilidades ecológica e economicamente interessantes: o tratamento de efluentes com maior qualidade e a concomitante produção de biomassa, capaz de gerar produtos de valor comercial (RAWAT *et al*., 2011; CHAN *et al*., 2011). A tabela 2 sintetiza os valores de produtividade e para remoção de nutrientes encontrados na revisão de literatura aqui apresentada.

Sawayama e colaboradores (1998) demonstraram a capacidade da microalga *Botryococcus braunii* em crescer bem em efluente do tratamento secundário de ETE no Japão. Neste trabalho foi observada a redução dos nutrientes (NO_3 e PO_4, 25% e 51% respectivamente), produção de biomassa a 196mg L^{-1} por semana e o alcance de 64% de peso úmido em óleo. Ainda com a espécie *B. braunii*, Órpez e colaboradores (2009), conseguiram bons resultados utilizando também efluentes de tratamento secundário como única fonte de nutrientes. O teor de lipídios, como esperado em cultivos com falta de nutrientes (1,49 mg L^{-1} d^{-1}), foi mais elevado (18 %) em comparação com o uso de meio sintético (1,19 mg L^{-1} d^{-1}).

Efluentes de lagoas de estabilização (tratamento aeróbio) foram utilizados em ensaios com *Scenedesmus obliquus* e *Chlorella vulgaris*, para averiguação da taxa de remoção de nutrientes. Os resultados mostraram valores de remoção de 100% para amônia e 59% para fosfato (o efluente com valores iniciais de 36 e 111 mg L^{-1}, respectivamente). O trabalho mostrou a possibilidade de tratamento de efluentes em sistemas de cultivo fechados e de volume médio, 2 e 30 litros (GONZÁLEZ;CANIZARES;BAENA, 1997).

Martinez e colaboradores (2000) avaliaram efluentes do tratamento secundário (anaeróbio) com a espécie *Scenedesmus obliquus*. Foram estimados os valores de

produtividade (26mg L^{-1}) e taxa de remoção de nutrientes, em média para os parâmetros otimizados, a 98% (PO_4) e 100% (NH_4).

Bertoldi e colaboradores (2006) avaliaram o potencial de *C. vulgaris* em sistemas de cultivo com efluente de hidroponia. Embora o trabalho não foque a capacidade de remoção de nutrientes ou de biomassa, mostra o efeito de diferentes diluições do efluente (com água) na produção de lipídios (caracterizando os ácidos graxos) e carotenóides. Os resultados mostraram ausência de diferença significativa no teor lipídico e na maioria dos tipos de ácidos graxos, afetando apenas o teor de carotenóides totais.

Com objetivo de investigar a aplicação de microalgas em efluentes da suinocultura, pesquisadores espanhóis avaliaram o crescimento de *Chlorella sorokiniana* em conjunto com bactérias encontradas nos lodos ativados (provenientes de estações de tratamento). Os resultados mostraram uma resposta positiva no que se refere ao crescimento algal, remoção de amônia (mais forte por microalgas, 99%), fósforo (86%) e carbono orgânico total (mais eficaz por bactérias, 75%). Contudo, o efluente foi diluído diante da evidência de que em altas concentrações, as variáveis pH e teor de amônia inibiram o crescimento da microalga e colapsaram o sistema (GONZÁLEZ *et al*., 2008).

Ainda avaliando o potencial de efluentes provenientes de suinocultura, Godos e colaboradores (2009) obtiveram produção em biomassa (21 - 28g m^2) utilizando lagoas de estabilização de alta performance, com um consórcio de espécies de microalgas. Obtiveram também entre 70 – 90% de remoção de DQO e nitrogênio total, porém com menor remoção de fósforo (<10%). O efluente de suinocultura também teve que ser avaliado em diversas diluições, uma vez que bruto apresentou inibição de crescimento algal e colapso das lagoas, em concordância com os resultados de González (2008).

Kumar e colaboradores (2010) também avaliaram o efluente de suinocultura (em diversas diluições), com consórcio de espécies de microalgas, porém e resposta à inibição e baixa produção de lipídios, os forçaram ao uso de meios sintéticos em mistura aos efluentes, de modo a aumentar a produtividade e otimizar o processo em reatores fechados (melhor crescimento com 20mg L^{-1} de NH_4, apresentando redução de 60%, sem informar a biomassa final).

Tabela 2: síntese dos valores encontrados em pesquisa de literatura sobre a produção em lipídios e em biomassa (mg L^{-1} dia^{-1}) e em porcentagem de remoção de nutrientes (N, P e DQO). na = não analisado

Tipo de efluente	Epécie de microalga	Produtividade em biomassa (mg L^{-1} dia^{-1})	Produtividade em lipídios (mg L^{-1} dia^{-1})	Remoção de nutrientes	Referência
Água residuária - Tratamento secundário	*Botryococcus braunii*	28,0	1,49	25% (NO_3) 51% (PO_4)	SAWAYAMA et al., 1998
Água residuária - Tratamento secundário	*Scenedesmus obliquus*	na	na	100 % (NH_4) 59% (PO_4)	GONZÁLEZ et al., 1997
Água residuária - Tratamento secundário	*Chlorella vulgaris*	na	na	100 % (NH_4) 59% (PO_4)	GONZÁLEZ et al., 1997
Água residuária - Tratamento secundário	*Scenedesmus obliquus*	26	na	100 % (NH_4) 98% (PO_4)	MARTINEZ et al., 2000
Efluente de suinocultura	*Chlorella sorokiniana + bactérias de lodos ativados*	na	na	98 % (NH_4) 86% (PO_4)	GONZALEZ et al., 2008
Efluente de suinocultura	*Chlorella vulgaris*	na	na	60% (NH_4) 60% (PO_4)	KUMAR et al., 2010
Água residuária - Diferentes tratamentos	*Chlorella vulgaris*	na	na	60-80 % (NH_4) 50-90% (PO_4) 50-80% (DQO)	WANG et al., 2009
Água residuária artificial	*Scenedesmus sp.*	127	15,24	60 % (NH_4) 54% (PO_4)	VOTOLINA et al., 1999
Água residuária bruta e artificial	*Chlorella vulgaris*	na	10%	90 % (NH_4) 60% (PO_4)	RUIZ-MARIN;ESPINOZA;STEPHENSON et al., 2010
Água residuária bruta e artificial (mixotrofia)	*Chlorella vulgaris*	102	22	na	PÉREZ-GARCIA et al. 2010
Efluente de suinocultura (mixotrofia)	*Chlorella pyrenoidosa*	30	6,3	90 % (NH_4) 60% (PO_4) 50% (DQO)	WANG et al., 2012
Água residuária - Tratamento primário	*Chlorella vulgaris*	950	na	99 % (NH_4) 99 % (PO_4) 50% (NO_3)	RUIZ et al., 2011
Água residuária artificial	*Chlorella vulgaris*	350	147	96 % (NH_4) 97% (PO_4) 86% (DQO)	FENG et al., 2011

Wang e colaboradores (2009) apresentaram resultados mais completos sobre o crescimento de uma cepa de *Chlorella* sp. em efluentes domésticos tratados. Foram utilizados efluentes do pré-tratamento, decantador primário, tratamento aeróbio e efluente da secagem dos lodos. Como medida de crescimento foi avaliada apenas a taxa de crescimento, que apresentou os seguintes valores para os respectivos efluentes: 0,41, 0,42, 0,34 e 0,94. As taxas de remoção de nutrientes foram, seguindo a mesma ordem, as seguintes: 82, 74, 62 e 78% (N), 83, 90, 47 e 85 (P) e 50, 56, 0 e 83 (DQO). Os resultados mostraram correlação com a disponibilidade de nutrientes, apontando o centrate como efluente de melhor desempenho. Contudo, o excesso de nutrientes não pode ser removido em níveis aceitáveis quando aplicado bruto ao cultivo de *Chlorella* sp. Outras amostras de efluentes mostraram necessidade de suplementação de nutrientes.

Meios artificiais que simulam a composição de efluentes domésticos também foram testados por Feng e colaboradores (2011). Neste trabalho com *Chlorella vulgaris* foi calculada uma produção de 0,89 g L^{-1}, com 42% de lipídios. Ressalta-se que os autores utilizaram um efluente sintético que continha, além das formas inorgânicas de N (78 mg L^{-1} de NH_4) e P (18 mg L^{-1} PO_4), glicose como fonte de carbono orgânico, que pode ser utilizado em mixotrofia pela *Chlorella* (LEE, 2004). Os autores encontraram percentagens de remoção de nutrientes nos valores de 86, 97 e 96% para DQO, NH_4 e P total, respectivamente.

Voltolina e colaboradores (1999) também avaliaram meios artificiais que simulam efluentes domésticos. Estimaram uma produção baixa de biomassa, 127mg L^{-1}, e de lipídios, 12% do peso seco. Para remoção de nutrientes foram observadas taxas máximas de >50% para PO_4 e >60% para NH_4.

Estudando efluente do tratamento secundário (anaeróbio), Ruiz e colaboradores (2011) encontraram valores de produção de biomassa próximos a 1,0 g L^{-1} para a espécie *Chlorella vulgaris*. Nestas mesmas condições as taxas de remoção de nutrientes se aproximaram de 100% (sem adição de nutrientes), resultando em valores removidos de >60mg L^{-1} NH_4 e 35mg L^{-1} de PO_4.

Ruiz-Marin, Mendoza-Epinosa e Stephenson (2010) avaliaram as espécies *Chlorella vulgaris* e *Scenedesmus obliquus* em efluente urbano tratado. Encontraram para a primeira

espécie, 60% de remoção de NH_4 e PO_4. Para a *Scenedesmus* foram encontrados valores de 70 e 60%. O trabalho não versa sobre a produtividade em biomassa, uma vez que controla o crescimento por medidas de densidade celular (células mL^{-1}). Aponta, contudo, melhor crescimento em efluentes domésticos artificiais em comparação com os efluentes brutos.

Sydney e colaboradores (2011) realizaram testes *screening* com diversas cepas de microalgas em efluentes do tratamento secundário (anaeróbio), focando os estudos subseqüentes com as espécies *Botryococcus braunii* e *Chlorella vulgaris*. Os testes foram feitos em reatores de 2 litros, com produção de biomassa estimada em 0,48g L^{-1} (36% de lipídios) e 0,64g L^{-1} (8% lipídios), respectivamente. As taxas de remoção de nutrientes foram de 80% (N) e 100% (P) para *Botryococcus* e 74% (N) e 100% (P) para *C. vulgaris*.

Na tentativa de aumentar a produtividade de sistemas baseados em microalgas, uma série de estudos foi desenvolvido para avaliar o metabolismo mixotrófico e heterotrófico. Nestas modalidades de nutrição as microalgas podem, respectivamente, realizar fotossíntese em conjunto com a absorção de fontes orgânicas de carbono ou exclusivamente consumir carbono orgânico na ausência de luz (LEE, 2004). A microalga diatomácea *Phaeodactylum tricornutum* foi extensivamente pesquisada com diversas fontes de carbono. Os resultados apontaram para a mixotrofia como alternativa extremamente viável como indutora da produção de ambos biomassa e lipídeos (CERON-GARCIA, 2006). Valores de produtividade de 2 a 16 g L^{-1} d^{-1} foram alcançados, um aumento médio de 8 a 10 vezes em comparação com cultivos autoróficos (CERON-GARCIA *et al*., 2000;2005;2006).

Pérez-Garcia e colaboradores (2010), estudando a espécies *Chlorella vulgaris* em ambos efluentes domésticos e meios sintéticos reportaram menor taxa de reprodução (de 0,78 para 0,72 d^{-1}) em cultivos com glicose em mixotrofia em comparação com as mesmas condições em autotrofia. Liang e colaboradores (2009), também estudando *C. vulgaris* em meio sintético reportaram um acréscimo de 10 vezes (biomassa) em culturas acrescidas de 100 mM de glicerina (102 mg L^{-1} d^{-1}) em comparação com culturas autotróficas (13 mg L^{-1} d^{-1}). Neste estudo a produtividade lipídica foi aumentada de 4 mg L^{-1} d^{-1} para 22 mg L^{-1} d^{-1} com adição da glicerina em comparação ao cultivo autotrófico.

Wang e colaboradores (2012) registraram valores de 30 mg L^{-1} d^{-1} em produtividade de biomassa e 6,3 mg L^{-1} d^{-1} em produtividade lipídica para *Chlorella pyrenoidosa* cultivada mixotroficamente em efluentes de suinocultura tratados anaerobicamente. Foram

alcançado conjuntamente valores de remoção de NH_4 >90%, DQO >50% e PO_4 >60% em efluentes co diluições de até 5 vezes em água deionizada.

Embora o conhecimento sobre cultivo de microalgas em águas residuárias venha sendo desenvolvido há pelo menos 50 anos, apenas na última década tem sido focado na produção de biomassa para energia. Dentre os trabalhos atuais sobre o tema, muitos focam a remoção de nutrientes, sem envolver produção de biomassa. É ainda maior a lacuna, entre os trabalhos recentes com microalgas em águas residuárias, referente à produção de metabólitos de interesse ou aproveitamento de biomassa para bioenergia.

3. OBJETIVOS

A presente proposta tem o objetivo geral de avaliar o potencial de efluentes domésticos urbanos como fonte alternativa de nutrientes para o cultivo de microalgas para geração de biocombustíveis.

3.1. Objetivos Específicos

- Produzir biomassa em águas residuárias domésticas como substrato alternativo com as espécies *Chlorella vulgaris* e *Botryococcus terribilis*;

- Redirecionar o metabolismo em microalgas como indutor de maior produção de biomassa e óleo para biocombustíveis;

- Demonstrar a capacidade de depuração de efluentes domésticos por microalgas, com resultante produção de biomassa;

-Contribuir para o desenvolvimento de pesquisas e formação de recursos humanos para a inclusão da biomassa microalgal na cadeia produtiva de biocombustíveis no Brasil, bem como para o desenvolvimento de tecnologias mais limpas e CO_2 neutras e mitigadoras.

4. RESULTADOS E PRODUTOS GERADOS

4.1. Cultivation of the green microalgae *Chlorella vulgaris* in treated urban wastewater: growth kinetics, nutrient removal, biomass production and carbon fixation

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ABSTRACT:

The demand for clean water is currently a worldwide priority. For this reason, further developments in the wastewater treatment sector are a strategic governmental responsibility. The traditional processes are considered inefficient for removing most of these nutrients. New treatment systems have been developed but there are still significant implications in regard to costs. Systems based on microalgae have been used for assisting in nutrient removal but limited advancement has been made in regard to obtaining valuable products from such practice. Microalgae systems are very versatile in regards to the potential products. Thus, it is very important not only to investigate algal toxic response and biomass yields when using such effluents, but also it is paramount to test distinct effluents. The aim of this work is to evaluate the effect of different Wastewater treatment plants (WWTP) effluents as a source of nutrients for producing biomass of the green microalgae *Chlorella vulgaris*. The experiments were carried out in 3 trials, to evaluate (i) the effect of different effluents from 2 WWTP, (ii) test the consortia between microalgae and bacteria from activated sludge in primary settler effluent and (iii) to evaluate the changes of the N/P ratio in the Centrate effluent. The experiments were carried out in Pyrex reactors (2L), under standard conditions. The biomass and nutrient evolution was accompanied. The final biomasses were evaluated by their content in C,H,N,S and P. *Chorella vulgaris* has presented a satisfactory growth in all tested conditions, with final biomass ranging between 50 to 150 mg per liter per day. As discussed, the range of the productivity values are similar to data showed elsewhere. Such findings indicate the feasibility of wastewater, as it is currently processed, for microalgae culturing. In all samples tested it was observed nutrient removal The majority has presented final concentrations below legal threshold: <10 mg L^{-1} for nitrogen, <1 mg L^{-1} for phosphorus and <125 mg L^{-1} O_2 for COD. The biomass composition (as C,H,N,S and P) was similar to the expected within the culture conditions and media composition. Supplementation of CO_2 is an important variable for biomass production and efficient nutrient removal. *Chlorella vulgaris* was capable of fixating from 56 to 242 mg per liter per day of CO_2. Artificial modification of the N/P ratio didn't increase biomass production neither nutrient removal. Such findings reinforce the suitability of treated urban wastewater as a nutrient source for microalgae cultivation.

Key-words: wastewater; *Chlorella vulgaris*; depuration; biomass production; CO_2 mitigation

1. Introduction

The demand for clean water is currently a worldwide priority. For this reason, further developments in the wastewater treatment sector are a strategic governmental responsibility. On the other hand, in this decade, the main challenge of a Wastewater Treatment Plant (WWTP) is not only to produce reusable clean water, but it is also to find resources for supporting those new developments. In 2009, the USA government alone devoted 4 billion dollars in loans for investments in infrastructure and rehabilitation of WWTPs (Malarkey & Adducci, 2011). WWTPs commonly offer a treatment composed of several stages based on physical, chemical and biological methods (Carey & Migliaccio, 2009). Nevertheless, they often remove only a fraction of the total nitrogen and phosphorous (Rawat *et al.*, 2011). The traditional aerobic/anaerobic processes, for instance, are considered inefficient for removing most of these nutrients (Chan et al., 2011).

New treatment systems have been developed in order to improve depuration of water, but there are still significant implications in regard to costs. For instance, improved technology for removing nutrients requires an increase in energy consumption of about 60 to 80% (Lam & Lee, 2011). The main target of such systems has been to improve removal of nutrients from effluents, during WWTP secondary and tertiary treatment (Lam & Lee, 2011). A novel and revolutionary approach, however, would be to combine wastewater treatment with the production of renewable energy, such as biofuels. The combination of these processes can significantly affect quality of the treatment and, at the same time, produce potential revenue capable of supporting new and sophisticated technologies. This innovative approach may represent a more sustainable alternative for this sector. The cost of treatment is counterbalanced with the production of valuable products, which in turn can also cause a significant economic impact in the society as a whole (Rawat *et al.*, 2011). However, such studies are still limited (Chan *et al.*, 2011). Biological processes capable of combining treatment with energy production have been known for decades (Park et al., 2011). On the other hand, as living systems, they are directly dependent on natural biodiversity and on a standardized process for production and control. These are often very

difficult to achieve. In other words, there is very limited knowledge in regards to the biological potential and process operation of such living systems. The goal of this research is to contribute with the advancement of this area.

Systems based on microalgae have been used for assisting in nutrient removal (Oswald, 1957; Benneman, 1980), but limited advancement has been made in regards to obtaining valuable products from such practice (Larsdotter, 2006; Park *et al*., 2011). Microalgae systems are very versatile in regards to the potential products. Biomass from microalgae can be used to generated biodiesel (from oil), bioethanol (starch fermentation), bio-kerosene (liquid hydrocarbons), bio-plastics (biopolymers), bio-hydrogen, biogas (methane from biomass digestion) and chemicals derivates (Suchardt, 1998; Chisti, 2007). Therefore, the main goals of current developments have been based on bioprospecting species and develop cost-effective systems favoring high yields at low maintenance costs (Ruiz-Marin *et al.*, 2010). In an elementary statement, the costs of microalgae-based systems are often identified as 50% for biomass production and another 50% which is spent on downstream processes such as: harvesting, drying and oil extraction (at least 30%). The costs of biomass production can be significantly reduced (at least 30%) if another source of nutrients is used instead of the expensive artificial amendments made in synthetic media (Sheehan et al., 1998). WWTP effluents are, therefore, identified as ideal common sources of such nutrients (Larsdotter, 2006; Rawat *et al*., 2011). On the other hand, WWTP effluents are complex effluents and they vary substantially in nutrient composition at distinct stages of the treatment (i.e. WWTP effluents from secondary and tertiary treatment). Thus, it is very important not only to investigate algal toxic response and biomass yields when using such effluents, but it is also paramount to test distinct effluents which are commonly obtained at different stages of wastewater treatment process. The aim of this work is to evaluate the effect of different WWTP effluents as a source of nutrients for producing biomass of the green microalgae *Chlorella vulgaris*. This is an organism known for being capable of producing byproducts, which are valuable for the biofuel industry (Nascimento *et al*., 2011); and it has also already been used to improve effluent treatment in WWTP (Kumar et al., 2010). Thus, this research is targeting an ideal organism which is able to bridge the gap

between improving nutrient removal of WWTP systems and the production of renewable energy.

2. Material and methods

2.1. Microorganism and culture conditions

Chlorella vulgaris, strain SAG211-12, acquired from Culture Collection of Algae/Gottingen University (Germany), and was used for the experiments. The inoculum and trials were prepared using Combo medium (Kilham *et al.*, 1998), constant aeration (1v/v/m) with filtered air (0.2 μm), photoperiod of 14:10 light:dark cycles under 150μmols/m^2/s of luminance and incubated at a temperature of 20±1°C.

2.2. Experimental set-up

The first trial was design to evaluate the effect of different wastewater effluents from two wastewater treatment plants (WWTP) from the Provinces of Cadiz and Sevilla, Spain. The effluents from different treatment processes were: Pretreated effluent (from WWTP 1 and 2), anaerobic treated wastewater (WWTP 2), disposing effluent (WWTP 1 and 2), the effluent from primary settler (EPS) and the centrate (from drying sludge).

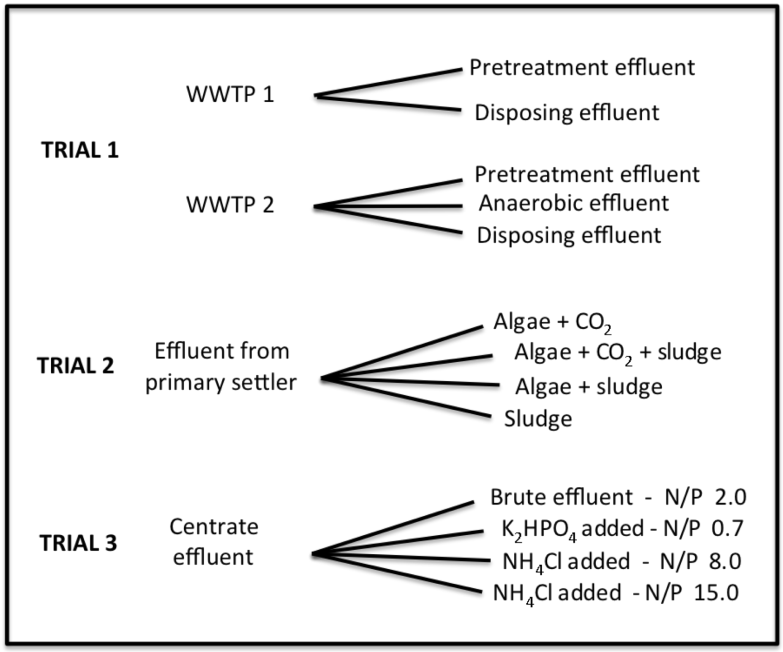

Figure 1: Experimental design of the trials carried out in the present work. The acronyms are wastewater treatment plants (WWTP), nitrogen (N) and phosphorus (P).

The second and third trials were carried out in two stages in order to attend two objectives: (i) testing the growth of microalgae in a consortium with inoculated bacteria from activated sludge (Figure 1) and (ii) to evaluate the effects of using the centrate as medium at distinct N/P ratio (Figure 1).

2.3. Analytical methods

The reactors were monitored daily assessing optical density (OD 680nm), pH, temperature (^{o}C) and total suspended solids (TSS mg/L) (APHA – Standard Methods, 1995). Biomass was recovered by centrifugation at 4200 rpm, fallowed by freezing and lyophilization. Biomass was analyzed by the composition of C,H,N,S and P in a automatic combustion chromatographer (CHNS Leco 932).

The wastewater samples were analyzed at the beginning and end of the experiments by their composition of ammonium (N-NH_4), nitrate (N-NO_3), nitrite (N-NO_2), total nitrogen (TN), phosphate (P-PO_4) and total phosphorus (TP), using an ionic chromatographer and analytical determination kits (Merck®) in the case of total phosphorus and total nitrogen. It

was also analyzed by initial pH, conductivity, turbidity, COD (chemical oxygen demand) and total suspended solids (TSS).

2.4. Data analysis

The kinetics of growth were resolved in SigmaPlot® v.12 using a sigmoidal curve. The software is also equipped with a statistical package for testing the fitness of the model used. The results are expressed as probability ($p<0.05$).

The kinetic parameters were also crosschecked using linear regression of the exponential phases. The approach was applied on the experimental data and their Ln transformed values for calculating productivity and growth kinetics, respectively.

In order to compare the parameters estimated by the models it was carried out a t-test, for comparison of two groups. For multiple groups comparisons it was used an analysis of variance (ANOVA). Both were applied within the software Graphpad instat v.3 at the significance level of 0,05.

3. Results and discussion

3.1. Biomass production

The obtained growth curves are shown in Figure 2. Figure 2A shows the response of *Chlorella vulgaris* SAG211-12 exposed to wastewater samples from different stages of the treatment process. The concentration of nutrients at each stage is shown in Table 1. The microalgae presented a typical microbial growth in all treatments. Also it was observed a linear correlation between productivity and nitrogen concentration, which showed a R^2 of 84% with 5 points. The efficiency of each treatment followed this order: pretreatment

WWTP1 > Anaerobic treatment WWTP2 > pretreatment WWTP2 > effluent WWTP2> effluent WWTP1, reaching from 61 to 150 mg per liter of biomass in the cultures. Since nitrogen is the main nutrient for algae cultivation (after carbon), the values of productivity are in agreement with those reported in the literature for *C. vulgaris* cultured in wastewater. The present results are similar to the ones reported by Feng and coworkers (2011). They reported values from 44 to 147 mg L^{-1} d^{-1} for *C. vulgaris* grown in artificial wastewater (78 mg of NH_4 and 18 mg of PO_4, per liter). An average value of 18 g m^{-2} d^{-1} for *Chlorella* sp. is reported for wastewater treatment. Other species from the *Chlorella* genus showed a range of 15 to 35 g m^{-2} d^{-1} (Park *et al.*, 2011).

The applied model describes a substrate dependent growth and represents the increase of biomass and its growth limitation in batch culturing systems. To measure the accuracy with which the referred model fit the experimental data, the parameter p-value was estimated (table 3). The different wastewater samples have presented similar results and p-values below 0.01, considered extremely significant. Such findings show that the experimental data have fitted to the applied model.

Growth curves results using the effluent from the primary settler with different conditions are shown in Figure 2B. Highest growth rates were observed with injection of CO_2 mixed with the air (EPS I). Results from other authors working in similar conditions corroborate the present data (Sawayama *et al*., 1995; Ruiz-Marin *et al*., 2010; Park *et al*., 2011). It was observed a lower productivity ($p<0.05$) with the CO_2 addition and in the presence of the same amount of inoculum of activated sludge. The productivity in this condition was 78 mg L^{-1} d^{-1} compared to the 84 mg L^{-1} d^{-1} for microalgae incubated alone. The microalgae cultivated with activated sludge without CO_2 (EPS III) presented a productivity of 66 mg L^{-1} d^{-1}. It was a lower value ($p<0.05$) when compared to the condition with addition of CO_2, demonstrating the importance of CO_2 supplementation. The CO_2 addition is an advantageous possibility since it is an environmental problem and microalgae can fixate it as biomass, which can be used for fossil fuels substitution (Rawat *et al*., 2011; Chisti, 2007; Schenk, 2008). Finally, the activated sludge alone (EPS IV) comparatively

presented a smaller development of 25 mg L^{-1} d^{-1}, $p < 0.01$. However, the aim of such reactor was not biomass production, but to evaluate the development of the system and the role of the sludge.

The productivity values presented correlation with the biomass produced (EPS I >EPS II> EPS III> EPS IV). The kinetic parameter μ (specific growth rate) showed similar values between the conditions inoculated with microalgae (0.13, 0.13 and 0.12). The main comparison to be made in this analysis is between EPS II and EPS III. The results clearly show the importance of CO_2 supplementation towards achieving higher biomass yields (1040 against 680 mg per liter). It is also possible to notice in the EPS I, standard condition for microalgae growth, which presents CO_2 addition and the highest biomass yields (1389 mg per liter).

While in C it can be seen the analysis with the Centrate wastewater with artificially different N/P ratios. It is worth noticing the latency time of about ten days, both in the original and the artificially modified Centrate. Such large acclimation phase can be explained by the high values of ammonium in the medium. Some authors have found problems in cultivating microalgae in media with high concentrations of ammonium (González *et al*., 2008; Kumar *et al*., 2010; Godos *et al*., 2009). Nevertheless, it was possible to observe the classical microbiological evolution in all tested conditions. Statistical analysis (table 5) showed that the used model was appropriated. The highest yield was observed in the condition III (N/P = 8.0), significantly different ($p < 0.05$) from the original one with N/P of 2.0 (96 against 84 mg L^{-1} d^{-1}). The other tested conditions did not show a linear correlation between the N/P ratio and biomass productivity (R^2 equals 0,14 with four points). Even though the centrate III presented higher productivity than centrate II, the difference of only 12 mg L^{-1} d^{-1} may point towards to be unnecessary to alter artificially the N/P ratio of the centrate effluent to increase biomass yields. This also highlights the feasibility of the centrate as a suitable nutrient source for production of microalgae biomass. Wang and coworkers (2009) tested a *Chlorella* sp. strain in the Centrate effluent and found the highest μ (day^{-1}) when compared to other wastewater effluents (0.95 against 0.41). Since the

specific growth rate (μ) was calculated using the optical density in this work it is impossible to compare with the present results. Kong and coworkers (2010), using *Chlamydomonas reinhardtii* in 15 liters bioreactors, reached a 2g L^{-1} final biomass in the centrate effluent. Those studies reinforce the present data for the feasibility of the centrate wastewater as nutrient source for microalgae cultivation.

3.2. Nutrient removal

In all conditions tested it was observed a reduction of nitrogen, phosphorus and COD (chemical oxygen demand). The European Directive 98/15/EC establish a nutrient removal percentage in the wastewater treatment of 80% for phosphorus (up to 1mg L^{-1}), between 70-80% for nitrogen (up to 10mg L^{-1}) and 75% (or up to 125 mg L^{-1} O_2) for COD. The most restrictive values are for plants operating for over 100.000 habitants equivalent.

In the first trial of experiments, N and P removal ratios were above 90%, (Table 2), with the exception of the experiments with disposing effluent (WWTP1), which showed a reduction of 60% for nitrogen. This condition was the only sample that did not reduce the nitrogen concentration below 10mg L^{-1} (only 14,6), statistically different from the other treatments in the same trial ($p<0.05$). It is important to highlight that in this treatment it was observed the lowest biomass production of all (796,1 mg L^{-1}), what explains the remaining nitrogen. Regarding to phosphorus removal, in all samples it was observed the reduction to concentrations below 1 mg L^{-1} (all conditions with removal above 92% and significantly equals, $p>0.05$). COD removal was low (from 25 to 50%), but such results were expected since microalgae are not primarily efficient in removing this parameter in autotrophic conditions (Lee, 2004). For such purpose often, bacterial systems are applied (Rawat *et al*., 2011). However, *Chlorella vulgaris* is known for also growing mixotrophic and heterotrophically, i.e. uptaking also the organic carbon present in the substrate, which explains the observed values of COD removal in the present work (Li *et al*., 2011; Bhatnagar *et al*., 2011; Lee, 2004).

The second trial tested the association between microalgae and the bacteria from activated sludge (with effluent from primary settler), which did not affect biomass productivity ($p>0.05$). A distinct effect was also not observed for nitrogen and phosphorus removal ($p>0.05$). However, it was observed an increase ($p<0.05$) in COD efficiency removal (from 44 to 82% with the sludge), without a significant reduction in N and P uptake. COD removal was higher without CO_2 addition (82, 25%). On the other hand, in such conditions, biomass yields were 50% lower. Such data are in agreement with other results regarding association between microalgae and bacteria from wastewater treatment processes. Results from Kumar and coworkers (2009) showed only a 60% reduction in total ammonium and 70% in phosphorus, using *Chlorella vulgaris* grown in effluent from anaerobic digestion of piggery manure. Also with piggery effluents, Godos and colleagues (2009) found removal rates of 88% for total nitrogen (mainly ammonium) and 76% for COD, using a consortium of microalgae species in high rate stabilization ponds. González and coworkers (2008), using the species *Chlorella sorokiniana* reached 99%, 86% and 75% for removal of, respectively, NH_4, PO_4 and COD growing in diluted manure.

The trials with the centrate were directly affected by different N/P ratios (table II). The highest nitrogen removal rate were observed in the original condition (N/P = 2,0), followed by centrate I (N/P =0,7). At higher ratios, the removal of nitrogen was significantly lower. The elevated level of ammonium in the medium can explain such findings. The excess of this nutrient could probably have inhibited *C. vulgaris* metabolism, which explains both reduced nutrient consumption and biomass production (Godos *et al*., 2009; Baumgarten *et al*., 1999, Kumar *et al*., 2010). Wang and coworkers (2009) have reported removal percentages of 78% (N), 85% (P) and 83%(COD) for Chlorella sp. cultivated in the Centrate. In none of the four tested conditions the most restrictive threshold of European directive could be reached (the original condition came close with 10,5mg L^{-1}). However, it is important to highlight that the conditions with 0.7 and 2.0 N/P ratios have reached an average of 90% nitrogen reduction.

Phosphorus removal exhibited a similar pattern. An increase in N:P ratios reduced phosphorus uptake, as observed in the condition III (N:P = 8,0) and it did not significantly change the efficiency in the condition IV (N:P = 15,0). Lower ratios resulted in a decrease of phosphorus uptake. In all cases, reductions were not sufficient to attend the European directive. It was observed a phosphorus concentration about 40 times over the legal threshold (42, 5 in the best and original condition).

Wang and coworkers (2009) evaluated *Chlorella* sp. with the centrate effluent (N:P:COD of 131:201:2250mg L^{-1}, N/P = 0.6) and found removal values of 78, 85 and 83% for respectively N, P and COD. Those values are in agreement for nitrogen and COD removal in the present work for similar N/P ratio (0.7). Kong and coworkers (2010), studying *Chlamydomonas reinhardtii* in the centrate showed removal values of >80% for nitrogen and phosphorus (within a N/P of 3.4). High ratios between nitrogen and phosphorus, about 30:1, suggest P-limitation, whereas low ratios of about 5:1 suggest N-limitation (Larsdotter, 2006). Since wastewater often exposes the algae to nutrient concentrations of up to three orders of magnitude higher than under natural conditions, growth is more likely limited by carbon and light (Larsdotter, 2006).

COD removal correlated negatively with increasing N/P ratios (R^2 equals 0,71 with four points). It should be highlighted that the initial COD concentration was very high (675 mg L^{-1}) in the experiments with high nitrogen addition, when compared to other treatments (between 90 and 160 mg L^{-1}). The former experiment showed a better COD uptake efficiency. The trials using Centrate I have generated a resultant effluent that attended the EU normative, once it achieved over 75% removal of COD and final values of N and P below 10 and 1mg L^{-1}, respectively. It is an evidence of the possibility to treat the centrate effluent even tough it presents very high COD values. It is well established, for microalgae-based systems, that effluents with high values of COD (over 500 mg L^{-1}) may cause inhibition of microbial growth (Godos *et al.,* 2009). This forces, in many cases, the operators to dilute the effluents before using for cultivation, increasing the water footprint of the enterprises (Kumar *et al*., 2010; González *et al*., 2008).

3.3. Elementary analysis

The results of elementary analysis of biomass as C, H, N and S are shown in Figure 3. Regarding to carbon, all samples showed the expected concentration of average 50% of dry weight. These values where found within the expected range of 40 to 60% (Grobelaar, 2004). This shows the suitability of wastewater for *C. vulgaris* nutrition. The results with effluent from primary settler (EPS) were coherent with the culturing conditions. The highest carbon concentrations were observed in EPS III (sludge with microalgae without CO2) and IV (sludge alone). This value is explained by the sludge capacity of up taking carbon (Kargi & Uygur, 2002). In the case of the consortium with *C. vulgaris* it is well established the role of microalgae in supplying sludge with sufficient oxygen for its maintenance (Rawat *et al*., 2011). The two first conditions were without sludge (I) and sludge plus microalgae with CO2 supplementation, which favors more the autotrophic growth.

The results with the centrate showed a positive correlation between carbon percentages and COD removal rates (lower in the higher N/P ratios). This highlights a better carbon uptake at higher C/N ratios by *Chlorella vulgaris*.

The second most important nutrient for algal growth is nitrogen, usually found between values of 1 and 10% (Grobbelaar, 2004). Nitrogen content was observed within this expected range in all reactors in this trial. The phosphorus percentage is expected to be less than 1%, which was found in the two first trials. However, the reactors with centrate wastewater (from II to IV) presented values slightly above 1% (1.38; 1.15 and 1.09%). Those results can be associated with the original high concentration of P.

The first reactor (N/P = 0,7) presented a very high concentration of P. Such results are explainable by the metabolic shift imposed to the microalgae with the N/P ratios modifications, especially within the first conditions that received almost 5 times more phosphate than the other samples (which received nitrogen as NH4). Regarding to hydrogen

and sulfur, they were also found in values between the ones predicted by literature (2,9 to 10,0% and 0,15 to 1,6% respectively) (Grobelaar, 2004).

3.4. Carbon dioxide fixation rates

Microalgae can fixate carbon dioxide as biomass more effectively than terrestrial plants (Shuchardt, 1998; Chisti, 2007; Schenke, 2008). The carbon content (as percentage of dry weight) in the final biomass ranged from 43 to 56 % in the current trials. Such data shows that even when cultivated in different wastewater samples the carbon content of the final biomass is within the expected values of 40-60% (Grobelaar, 2004). Microalgae are known to assimilate carbon dioxide more effectively than plants and for each Kg of biomass, microalgae can assimilate up to 1.5 -2.0kg of CO_2 within their biomass (Brown & Zeiler, 1993).

Several authors apply an equation that correlates the biomass productivity with the biofixation rate of a specific microalgae (Chisti, 2007). The equation was derived using the general composition of the biomass of microalgae and it is expressed as:

$$Rco_2 = 1.88 \times P_B$$

Where, Rco2 is the maximum CO_2 fixation rate (mg CO_2. L^{-1}. d^{-1}), P_B is the biomass productivity and 1.88 is a constant.

Although well established, this equation considers a carbon content in biomass of 50%. Taking the actual carbon content into consideration would increase the accuracy of CO_2 fixation rates. Since such data were available, it was possible to calculate the experimental CO_2 fixation rate (Y CO_2), expressed as follows:

$Y\ co_2 = P_B \times (\%C/100) \times (PM\ co_2/PA_C)$

Where, P_B is the biomass productivity, %C is the carbon content in biomass, PM co_2 = 43,99 (carbon dioxide molecular weight), and PA_C = 12 (carbon atomic weight).

Table 6 shows both theoretical and experimental carbon dioxide fixation rates. The theoretical equation (Chisti, 2007) takes into account a biomass with 50% o carbon (as dry weight). Meanwhile, the experimental model uses the actual carbon concentration in biomass. Since *Chlorella vulgaris* presented carbon content in average circa of 50%, it explains the similarity between the results of both two forms of calculation. *C. vulgaris* could fixate from 56 to 242 mg $L^{-1}d^{-1}$ in the trials.

The comparison between the theoretical and experimental values shows the applicability of Chisti (2007) equation in assessing the carbon fixation rate, especially in the absence of elementary analysis. The application of the second equation may represent a possibility of higher accuracy, suggested specially in experimental conditions, which may generate carbon percentages in biomass higher than 50%.

4. Final remarks

Chorella vulgaris has presented a satisfactory growth in all tested conditions, with final biomass ranging between 50 to 150 mg per liter per day. As discussed, the range of the productivity values are similar to data showed elsewhere. Such findings indicate the feasibility of wastewater, as it is currently processed, for microalgae culturing.

In all samples tested it was observed nutrient removal (measured by nitrogen (N), phosphorus (P) and chemical oxygen demand (COD) uptake). The majority has presented final concentrations below legal threshold: <10 mg L^{-1} for nitrogen, <1 mg L^{-1} for phosphorus and <125 mg L^{-1} O_2 for COD.

The biomass composition (as C,H,N,S and P) was similar to the expected within the culture conditions and media composition.

Supplementation of CO_2 is an important variable for biomass production and efficient nutrient removal. *Chlorella vulgaris* was capable of fixating from 56 to 242 mg per liter per day of CO_2.

Artificial modification of the N/P ratio didn't increase biomass production neither nutrient removal. Such findings reinforce the suitability of treated urban wastewater as a nutrient source for microalgae cultivation.

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6. References

APHA. Standard Methods for the Examination of Water and Wastewater. **American Public Health Association,** Washington, DC. 1995

BAUMGARTEN, E; NAGEL, M; TISCHNER, R. Reduction of the nitrogen and carbon content in swine waste with algae and bacteria. **Appl Microbiol Biotechnol**, v. 52, p. 281-284, 1999.

BENNEMAN,JR; MIYAMOTO, K; HALLEBECK, PC. Bioengineering aspects of biophotolysis. **Enzyme and Microbial Technology**, v. 2, p.103–111, 1980.

BHATNAGAR, A *et al*. Renewable biomass production by mixotrophic algae in the presence of various carbon sources and wastewaters. **Applied Energy**, v. 88, p. 3425–3431, 2011.

BROWN, L. M.; ZEILER, K. G. Aquatic biomass and carbon dioxide trapping. **Energy Conversion and Management,** v. 34, p.1005-10013, 1993.

CAREY, RO; MIGLIACCIO, KW. Contribution of Wastewater Treatment Plant Effluents to Nutrient Dynamics in Aquatic Systems: A Review. **Environmental Management**, v. 44, p. 205–217, 2011.

CHAN, YJ *et al*. A review on anaerobic-aerobic treatment of industrial and municipal wastewater. **Chemical Engineering Journal**, v. 155, n. 1-2, p. 1-18, 2011.

CHISTI, Y. Biodiesel from Microalgae. **Biotechnology Advances**, v.25, p**.** 294-306,2007.

FENG, Y; CHAO, L; ZHANG, D. Lipid production of Chlorella vulgaris cultured in artificial wastewater medium. **Bioresource Technology**, v. 102, p. 101-105, 2011.

GODOS, I *et al*. Long-term operation of high rate algal ponds for the bioremediation of piggery wastewaters at high loading rates. **Bioresource Technology**, v. 100, p. 4332–4339, 2009.

GONZÁLEZ, C *et al*. Efficient nutrient removal from swine manure in a tubular biofilm photo-bioreactor using algae-bacteria consortia. **Water Science and Technology**, v. 59, n. 1, p. 95 - 102, 2008.

GRIFFITHS M.J.; HARRISON, S.T.L. Lipid productivity as a key characteristic for choosing algal species for biodiesel production**. Journal of Applied Phycology** ,v. 21,n.5,p. 493-507,2009.

GROBELAAR,JU. Algal Nutrition: Mineral Nutrition. In: Richmond, A (ed). **Handbook of microalgal culture: biotechnology and applied phycology**. Blackwell Publishing, 1 Ed., p. 97-115, 2004.

KARGI, F; UYGUR, A. Nutrient removal performance of a sequencing batch reactor as a funtion of the sludge age. **Enzyme and Microbial Technology**, v. 31, p. 842-847, 2002.

KILHAM, SS *et al.* COMBO: a defined freshwater culture medium for algae and zooplankton*.* **Hydrobiologia***,* v. 377, p. 147–159, 1998.

KONG, Q *et al*. Culture of microalgae *Chlamydomonas reinhardtii* in wastewater for biomass feedstock production. **Applied Biochemistry and Biotechnology**, v. 160, p 9-18, 2010.

KUMAR, MS et al. Influence of nutrient loads, feeding frequency and inoculum source on growth of *Chlorella vulgaris* in digested piggery effluent culture medium. **Bioresource Technology**, v. 101, p. 6012–6018, 2011.

LAM, MK; LEE, KT. Microalgae biofuels: a critical review of issues, problems and the way forward. **Biotechnology Advances**, in press, 2011.

LARSDOTTER, K. Wastewater treatment with microalgae – a literature review. **VATTEN**, v. 62, p. 31–38, 2006.

LEE, Y. Algal Nutrition: Heterotrophic Carbon Nutrition. In: Richmond, A (ed). **Handbook of microalgal culture: biotechnology and applied phycology**. Blackwell Publishing, 1 Ed., p. 116- 124, 2004.

LI, Y *et al*. Integration of algae cultivation as biodiesel production feedstock with municipal wastewater treatment: Strains screening and significance evaluation of environmental factors. **Bioresource Technology,** v**.**102, p. 10861 - 10867,2011.

MALARKEY, B; ADDUCCI, J. Water industry review: M&A market outlook 2011. **Boenning & Scattergood Report**. 17 p. 2011.

NASCIMENTO, IA *et al*. Screening microalgae strains for biodiesel production: lipid productivity and estimation of fuel quality based on fatty-acids profiles as selective criteria. **Bioenergy Resource**, in press, 2011.

OSWALD, WJ; GOTTAS, HB. Photosynthesis in sewage treatment. **American Society of Civil Engeneering**, n. 2849, p. 73-105, 1957.

PARK, JBK;CRAGGS, RJ; SHILTON, AN. Wastewater treatment high rate algal ponds for biofuel production. **Bioresource Technology,** v. 102, p. 35-42, 2011.

RAWAT, I *et al*. Dual role of microalgae: phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. **Applied Energy**, v. 88, p. 3411 – 3424, 2011.

RUIZ-MARIN,A;MENDOZA-ESPINOSA, LG; STEPHENSON, T. Growth and nutrient removal in free and immobilized green algae in batch and semi-continuos cultures treating real wastewater. **Bioresource Technology,** v. 101, p. 58-64, 2010.

SAWAYAMA, S;INOUE, S; YOKOYAMA, S. Continuous culture of hydrocarbon-rich microalga Botryococcus braunii in secondarily treated sewage. **Applied Microbiology Biotechnology**, v. 41, p. 729-731, 1994.

SCHENK, P. M. *et al*. Second generation biofuels: high efficiency microalgae for biodiesel production**. Bioenergy Research**, v.1,p.20-43,2008.

SCHUCHARDT, U; SERCHELI, R; VARGAS, R.M. Transesterification of vegetable oils: a review. **Journal of Brazilian Chemical Society,** v. 9, p. 199-210, 1998.

WANG, L *et al*. Cultivation of green algae Chlorella sp. in different wastewaters from municipal wastewater treatment plant. **Applied Biochemistry and Biotechnology**, v. 162, n. 4, p 1174-1186, 2010.

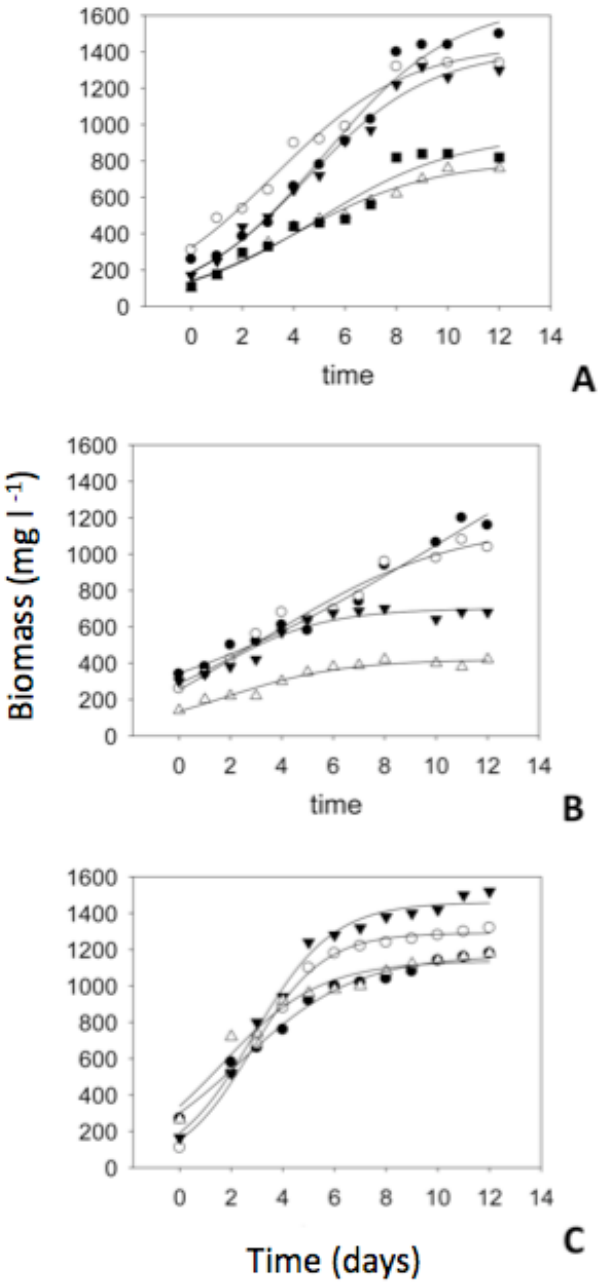

Figure 2: Growth curves of *Chlorella vulgaris*: Growth at: Pretreatment effluent WWTP1● Pretreatment effluent WWTP1○ anaerobic treated effluent WWTP2▼ Disposing effluent WWTP1n Disposing effluent WWTP1△ (A), effect of different cultivation conditions with effluent from primary settler (EPS): EPS I (microalgae + CO_2) ●, EPS II (microalgae+CO2+sludge) ○, EPS III (microalgae + sludge) ▼, EPS IV (sludge) △ (B), and growth in the centrate at different N/P ratios: I (N/P 0.7) ●, II (N/P 2.0)○, lll (N/P 8.0) ▼, IV (N/P 15.0)△ (C).

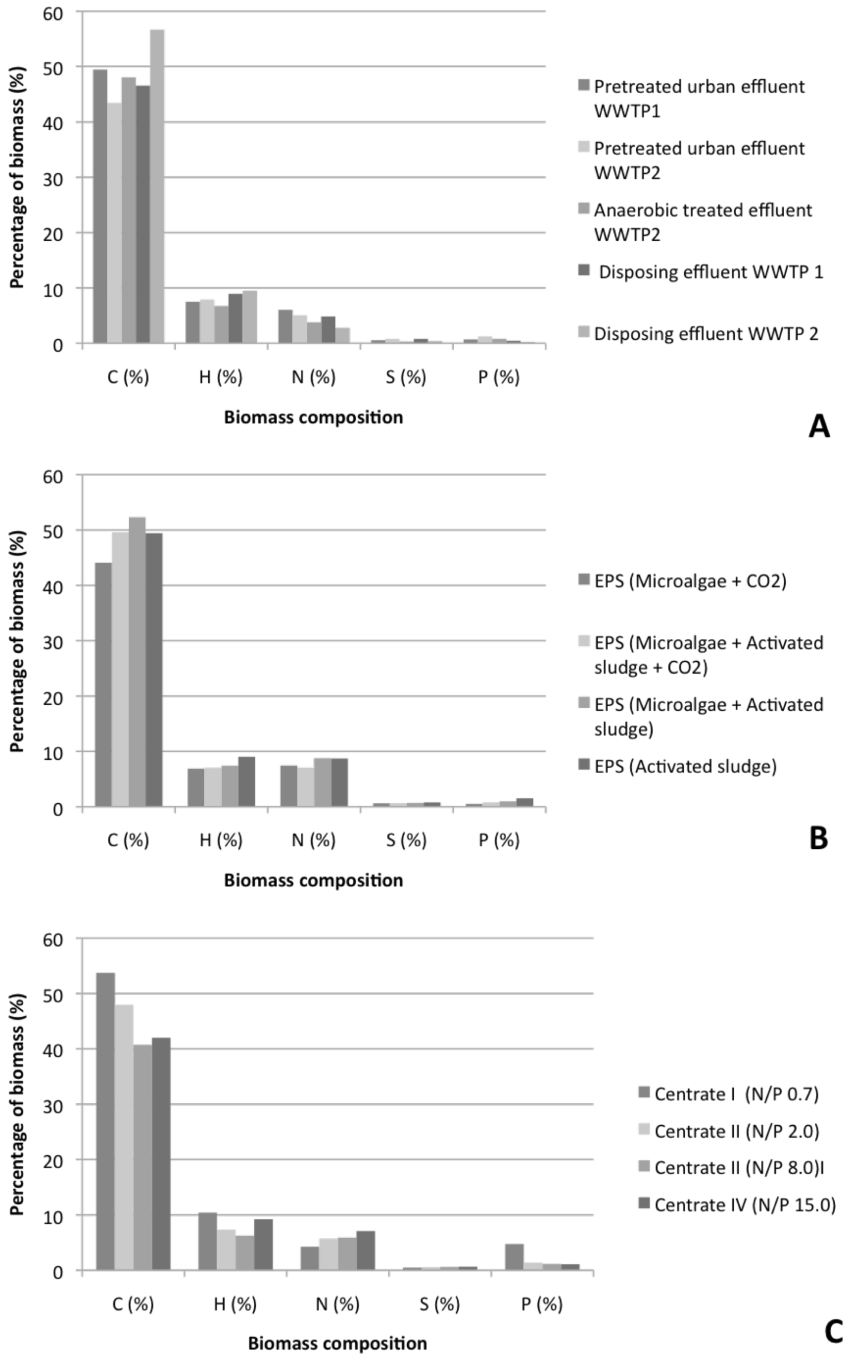

Figure 3: Chemical composition as C,H,N,S and P percentages in biomass: in different effluents from two WTP (A), effect of different cultivation conditions with effluent from primary settler (EPS)(B), and growth in the centrate at different N/P ratios (C).

Table 3: Results of the physical and chemical parameters analyzed in different wastewater effluents.

	Pretreated urban wastewater WWTP1	Pretreated urban wastewater WWTP2	Anaerobically treated wastewater WWTP2	Disposing effluent WWTP1	Disposing effluent WWTP2	Effluent primary settler (EPS-WWTP1)	Centrate I (WWTP2)	Centrate II (WWTP2)	Centrate III (WWTP2)	Centrate IV (WWTP2)
pH	8,0	8,1	7,8	7,5	8,1	7,2	6,9	7,1	6,5	6,5
Conductivity ($\mu S \cdot cm^{-1}$)	1112	1073	1141	995	700	961	1939	1939	1939	1939
SS ($mg \cdot L^{-1}$)	95	359	65	21	19	400	140	140	140	140
Turbidity (NTU)	113	183	95	8	7	55	73	73	73	73
COD (Filtered) (mg·L-1)	150	180	90	90	100	160	675	675	675	675
TP (Raw) ($mg \cdot L^{-1}$)	8,91 ± 0,38	8,81 ± 0,15	9,07 ± 0,24	2,72 ± 0,07	0,75 ± 0,04	5,08 ± 0,2	180,81	60,49 ± 1,7	60,49 ± 1,7	60,49 ± 1,7
TP (Filtered) ($mg \cdot L^{-1}$)	6,07 ± 0,26	5,93 ± 0,18	7,51 ± 0,16	2,38 ± 0,1	0,76 ± 0,02	3,20 ± 0,1	175,33	55,01 ± 1,0	55,01 ± 1,0	55,01 ± 1,0
$P\text{-}PO_4^{3-}$ (Filtered) ($mg \cdot L^{-1}$)	4,93 ± 0,06	4,89 ± 0,12	7,69 ± 0,39	2,12 ± 0,08	0,68 ± 0,01	1,7 ± 0,1	154,41	35,3 ± 1,5	35,3 ± 1,5	35,3 ± 1,5
TN (Raw) ($mg \cdot L^{-1}$)	88,47 ± 3,18	52,08 ± 9,48	64,14 ± 7,83	34,61 ± 1,25	9,79 ± 0,42	35,6 ± 1,0	130,1 ± 1,4	130,1 ± 1,4	471	909,9
TN (Filtered) ($mg \cdot L^{-1}$)	84,42 ± 2,65	41,96 ± 5,47	65,65 ± 1,43	36,44 ± 1,93	10,03 ± 0,33	33,9 ± 0,83	123,9 ± 1,5	123,9 ± 1,5	464,8	903,7
$N\text{-}NH_4^+$ (Filtered) ($mg \cdot L^{-1}$)	80,5 ± 6,62	39,55 ± 4,21	48,79 ± 5,46	23,34 ± 2,04	4,06 ± 1,23	30,6 ± 0,1	125,1 ± 2,1	125,1 ± 2,1	466,04	904,9
$N\text{-}NO_3^-$ (Filtered) ($mg \cdot L^{-1}$)	2,94 ± 0,6	< 0,5	< 0,5	7,23 ± 0,9	7,03 ± 0,23	< 0,5	< 0,5	< 0,5	< 0,5	< 0,5
$N\text{-}NO_2^-$ (Filtered) ($mg \cdot L^{-1}$)	0,18 ± 0,23	0,02 ± 0,01	0,02 ± 0,01	1,26 ± 0,21	0,27 ± 0,01	< 0,02	< 0,02	< 0,02	< 0,02	< 0,02
N/P	10	6	7	13	13	7	0,7	2	8	15

Table 4: Total nutrient removal (%) of *Chlorella vulgaris* in different wastewater treatment effluents.

	Pretreated urban wastewater WWTP1	Pretreated urban wastewater WWTP2	Anaerobically treated wastewater WWTP2	Disposing effluent WWTP1	Disposing effluent WWTP2	EPS I (Microalgae + CO2)	EPS II (Microalgae + Activated sludge + CO2)	EPS III (Microalgae + Activated sludge)	EPS IV (Activated sludge)	Centrate I (N/P 0.7)	Centrate II (N/P 2.0)	Centrate III (N/P 8.0)	Centrate IV (N/P 15.0)
TN removal (%)	90,05a	91,66a	98,48a	59,93b	90,03a	93,51a	92,04a	94,69a	55,75b	89,94a	91,49a	17,69b	6,55c
TP removal (%)	92,62a	93,48a	97,80a	92,36a	92,80a	92,81a	93,13a	92,19a	59,38b	19,98a	22,74b	10,56c	23,47b
COD removal (%)	40,00a	41,67a	50,00b	33,33c	25,00d	43,75a	75,00b	81,25c	84,38c	75,56a	56,30b	45,93c	42,22c

**Different letters show statistical significance ($p < 0.05$) by ANOVA test. Test carried out to compare the nutrient removal among the treatments within each trial.

Table 5: Verlhust kinetic parameters of *Chlorella vulgaris* grown in different wastewater treatment effluents. The model was fitted using STATISTICA software v.3 with a $R^2 > 0.80$ and a p-value < 0.05.

	Pretreated urban wastewater WWTP1	Pretreated urban wastewater WWTP2	Anaerobically treated wastewater WWTP2	Disposing effluent WWTP1	Disposing effluent WWTP2	EPS I (Microalgae + CO2)	EPS II (Microalgae + Activated sludge + CO2)	EPS III (Microalgae + Activated sludge)	EPS IV (Activated sludge) *	Centrate I (N/P 0.7)	Centrate II (N/P 2.0)	Centrate III (N/P 8.0)	Centrate IV (N/P 15.0)
X_O (mg SS·L^{-1})	108,0	110,0	107,0	100,0	108,0	140,0	206,0	200,0	140,0	107,0	110,0	105,0	105,0
X_M (mg SS·L^{-1})	1500,0	1340,0	1300,0	760,0	820,0	1160,0	1040,0	680,0	420,0	1180,0	1320,0	1520,0	1180,0
R^2	0,98	0,98	0,99	0,98	0,95	0,96	0,98	0,97	0,91	0,96	0,90	0,91	0,96
μ (day^{-1})	0,23	0,14	0,23	0,19	0,22	0,13	0,13	0,12	0,07	0,08	0,10	0,12	0,05
Productivity (mg SS·L^{-1}·day^{-1})	149,62a	120,17b	128,71c	61,83c	74,98d	84,45a	78,21b	66,07c	25,30d	64,00a	84,30b	96,48c	49,33d
Culture days	12	12	12	12	12	12	12	12	12	12	12	12	12

Abbreviations: X_o = Initial biomass; X_m = maximum biomass; μ = microalgae growth rate. *Model did not fit this condition, p-value > 0.05. **Different letters show statistical significance ($p < 0.05$) by ANOVA test. Test carried out to compare the parameters Xm and μ among the treatments within each trial.

Table 6: Comparison between the theoretical and experimental carbon fixation rates of *C. vulgaris* in the studied conditions.

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	Pretreated urban wastewater WWTP1	Pretreated urban wastewater WWTP2	Anaerobically treated wastewater WWTP2	Disposing effluent WWTP1	Disposing effluent WWTP2	EPS I (Microalgae + CO2)	EPS II (Microalgae + Activated sludge + CO2)	EPS III (Microalgae + Activated sludge)	EPS IV (Activated sludge)	Centrate I (N/P 0.7)	Centrate II (N/P 2.0)	Centrate III (N/P 8.0)	Centrate IV (N/P 15.0)
Rco_2 ($mg\ CO_2 \cdot L^{-1} \cdot d^{-1}$)	242,55a	186,55a	201,91a	112,13a	127,96a	145,46a	110,26a	56,47a	34,52a	157,03a	189,64a	136,38a	203,74a
Yco_2 ($mg\ CO_2 \cdot L^{-1} \cdot d^{-1}$)	233,93a	157,97b	189,22b	101,82b	141,40b	125,10b	106,67b	57,61a	33,28a	164,49b	177,36b	108,28b	166,89b

Abbreviations: Rco_2 = theoretical carbon fixation rate; Yco_2= experimental carbon fixation rate. **Different letters show statistical significance ($p < 0.05$) by t-test. Test carried out to compare the parameters Rco2 and Yco2 among the treatments within each trial.

4.2. Effect of glycerol on the metabolism of *Chlorella vulgaris* and *Botryococcus terribilis* cultivated in brute wastewater aiming biofuels production.

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Artigo elaborado com resultados das pesquisas conduzidas no Laboratório de Biologia Marinha (LABIOMAR/UFBA) no âmbito dos projetos CNPq (processos 574712/2008-9 e 551134/2010-0). A colaboração foi feita com parceria entre o LABIOMAR/UFBA e o Centro Andaluz de Ciência e Tecnologia Marinhas (CACYTMAR) da Universidade de Cádiz (UCA), Andaluzia/Espanha, com participação de pesquisador nas atividades desenvolvidas na UFBA/Brasil.

ABSTRACT:

With rapid population growth water withdrawals have tripled over the last 50 years, making the demand for clean water a worldwide priority. Wastewater treatment plants (WWTP) commonly offer a treatment composed of physical, chemical and biological methods. Nevertheless, they often remove only a fraction of the total nitrogen, phosphorous and chemical oxygen demand (COD). Systems based on microalgae have been used for assisting in nutrient removal and are very versatile in regard to the potential products. However, despite all studies accounted, there are very few data about their cultivation in wastewater with organic carbon addition and none involving glycerol. The aim of the present work was to evaluate the effect of glycerol addition to *Chlorella vulgaris* and *Botryococcus terribilis* grown in urban wastewater effluent. The experiments were carried out in Erlenmeyer flasks, under standard conditions and with glycerol addition (6.25mM, 12.5mM, 25.0mM and 50mM). The biomass and nutrient evolution was accompanied daily. The final biomasses were evaluated by their content in lipids, carbohydrates, protein and pigments. Both strains have presented a satisfactory growth in wastewater and with glycerol addition, with productivities from 47 to 111 mg L^{-1} d^{-1} for *Chlorella vulgaris* and from 55 to 165 mg L^{-1} d^{-1} for *Botryococcus terribilis*. Even tough no linear correlation could be established between glycerol concentrations and biomass production for both strains, it was observed a significant increase of biomass with 25 and 50 mM glycerol addition. This indicates the glycerol as a suitable carbon source for microalgae biomass production. Both strains have presented high values of nutrients removal efficiency above 70%, in average. Both strains presented expected values of carbohydrates and protein. The pigments (carotenoids and chlorophyll) have showed negative correlation with glycerol concentrations. The lipid productivity, even tough not correlated with glycerol availability, was significantly decreased with high concentrations (25 and 50mM). Such results suggest that glycerol addition is not recommended to produce lipids from both strains. Further research must be applied to other strains in order to verify the effect of glycerol under the lipid metabolism.

Key-words: Wastewater; *Chlorella vulgaris*; *Botryococcus terribilis*; mixotrophic

1. Introduction

With rapid population growth water withdrawals have tripled over the last 50 years, making the demand for clean water a worldwide priority (UNO, 2009). The water volumes, accounted as wastewater, were 70, 63 and 47 Km^3 for North America, Europe and Latin America, respectively (UNO, 2009). For this reason, further developments in the wastewater treatment sector are a strategic governmental responsibility. It also should be highlighted the current main challenge of a Wastewater Treatment Plant (WWTP) to produce not only reusable clean water, but also to find resources for supporting those new developments (Chan *et al*., 2011).

In 2009, the USA government alone devoted 4 billion dollars in loans for investments in infrastructure and rehabilitation of WWTPs (Malarkey & Adducci, 2011). WWTPs commonly offer a treatment composed of several stages based on physical, chemical and biological methods (Carey & Migliaccio, 2009). Nevertheless, they often remove only a fraction of the total nitrogen and phosphorous, which demands development in this area towards costs and operational optimization (Rawat *et al*., 2011).

Biological systems have been studied for achieving such needs (Park *et al*., 2011; Chan *et al*., 2011). However, traditional aerobic/anaerobic processes, for instance, are considered inefficient for removing most of these nutrients. New processes have been developed in the attempt to overcome those limitations, but have not yet extensively investigated to large scale and have few data available for their application to WWTP (Chan *et al*., 2011). It is also important to account the economical costs they would imply. For instance, improved nutrients removal would require an increase in energy consumption of about 60 to 80% (Lam & Lee, 2011). A new approach that could ally sustainable and economical feasibility would be to combine wastewater treatment with the production of renewable energy, such as biofuels (Park *et al.*, 2011). Systems based on microalgae have been used for assisting in nutrient removal (Oswald, 1957; Benneman, 1988), but limited advancement has been made in regards to obtaining valuable products from such practice (Larsdotter, 2006; Park et al., 2011). Microalgae systems are very versatile in regard to the potential products. Biomass from microalgae can be used to generated biodiesel (from oil), bioethanol (starch fermentation), bio-kerosene (liquid hydrocarbons), bio-plastics (biopolymers), bio-hydrogen,

biogas (methane from biomass digestion) and chemicals derivates (Suchardt, 1998; Chisti, 2007).

Another possibility is to take advantage of the metabolism of microalgae to achieve the necessary yields of a product of interest in alternative cultivation (Hu, 2008). Microalgae are known to be highly efficient autotrophic organisms (Richmond, 2004). However, several studies have reported their natural ability to grow mixotrophic and heterotrophic, i.e., in the presence of organic carbon sources (Haas & Tanner, 1974; Lee, 2004; Perez-Garcia *et al*., 2010). Among the studied species in such field, the *Chlorella* genus presented very interesting results (Wang *et al*., 2012; Liang *et al*., 2009) and since the 1970's is known for the presence of a hexose transport system, responsible for its resilient metabolism in natural environments (Haas & Tunner, 1974). The *Botryococcus* genus is studied since the 1950's for its ability to grow in highly eutrophized environments and its natural and remarkable capacity to produce liquid hydrocarbons similar to the ones found in petroleum (Metzger & Largeau, 1985; Benerjee *et al*., 2002; Dayananda *et al*., 2007). This last group has also been reported to have a mixotrophic growth (Tanoi *et al*., 2011; Tenaud *et al*., 1985). However, has also been reported as exclusive autotrophic (Weetall, 1985), which clearly demands more research in this matter.

Since organic carbon addition to biological systems represents an additional cost to the process (Chan *et al*., 2011), it is of most importance to find both inorganic and organic nutrients source to replace the expensive synthetic media. Wastewater (WW) has already been proved to be suitable for microalgae growth (Rawat et al., 2011; Park et al., 2011). Glycerol presents itself as a strong alternative carbon source, since it is currently been produced as a by-product of biodiesel trasesterification in significant amounts (Johnson & Taconi, 2007). In the year of 2010, the worldwide production of biodiesel was approximately 18 billion liters (bL), generating 1.8 bL of glycerol, considering a production rate of 10% of total volume (OECD, 2010). The Brazilian Biodiesel Program as accounted for the year of 2010 a volume of 2.4bL of biodiesel and consequent 240 million liters of glycerol production (ANP, 2010). Those data point towards the need to generate new markets for glycerol. For instance, the current glycerol market is around 900 thousand tonnes, way below the production levels (Johnson & Taconi, 2007).

Despite all studies accounted for the cited group of microalgae, there are very few data about their cultivation in wastewater with organic carbon addition and none involving glycerol with wastewater. For such matter, the aim of the present work was to evaluate the effect of glycerol addition to *Chlorella vulgaris* and *Botryococcus terribilis* cultures grown in urban wastewater effluent as source of nutrients.

2. Material and methods

2.1. Microorganism and culture conditions

In these experiments were used strains of *Chlorella vulgaris* Beijerick 1890 and *Botryococcus terribilis* Komaréck *1990*, stocked in the Microalgae Bank of the Marine Biology Lab (LABIOMAR) of Federal University of Bahia (Brazil). The inoculums and cultures were axenically prepared using CHU-13 medium (Largeau *et al*., 1985). Both inoculums and trials were kept under constant agitation (90 bpm) and aeration (with 2.5% CO_2 addition), photoperiod of 12:12 light:dark cycles, under 174µE/m^2/s of luminance and incubated at a temperature of 25±1°C.

2.2. Experimental set-up

The objective was to evaluate the effect of different concentrations of glycerol ($C_3H_8O_3$, analytical grade, CAS: 56-81-5) cultivated in wastewater. The effluent used was from the pretreatment (physical removal of large particles and fats) of a wastewater treatment plant (WWTP) located at the Province of Bahia, Northeast Brazil.

The trials were carried out in borosilicate Pyrex flask with 1 liter of total volume. The glycerol was added in the beginning of the experiment to 0.8 liter of wastewater effluent in the following concentrations: 6.25mM, 12.5mM, 25.0mM and 50mM. The highest concentration was selected by previous experiments, where it was observed an increase of

over two-fold in the viscosity of the media, causing problems in both cultivation and harvesting.

2.3. Analytical methods

The reactors were monitored assessing optical density (OD_{680nm}), pH, temperature (^{o}C), turbidity (NTU) and total suspended solids (g/L) (APHA – Standard Methods, 1995). Biomass was recovered by centrifugation at 4500x g, followed by freezing and lyophilization. Biomass was analyzed by the content (as percentage of dry weight) of protein (Bradford, 1990), carbohydrates (Rao Pragna, 1989), total lipids (Freeman et al., 1959), chlorophyll α and total carotenoids (Lee & Shen, 2004).

The wastewater samples were analyzed at the beginning and during the experiments by their concentration of ammonium ($N\text{-}NH_4$), nitrate ($N\text{-}NO_3$), nitrite ($N\text{-}NO_2$), total nitrogen (TN), phosphate ($P\text{-}PO_4$), chemical oxygen demand (COD, pH, conductivity, turbidity and total suspended solids (TSS)(APHA, Standard Methods, 1995). It was also analyzed the viscosity (initial and final) by the method of Stokes.

2.4. Data analysis

The kinetics of growth were resolved in SigmaPlot® v.12 using a sigmoidal curve. The software is also equipped with a statistical package for testing the fitness of the model used. The results are expressed as probability ($p<0.05$).

The kinetic parameters were also crosschecked using linear regression of the exponential phases. The approach was applied on the experimental data and their Ln transformed values for calculating productivity and growth kinetics, respectively.

To assess the differences among tested groups, it was carried out a parametric analysis of variance (ANOVA). As post-hoc test, it was used the Tukey's multiple comparison test (MCT). All analysis were carried out within the Graphpad Instat® software (v. 3, 2008), at the significance level of 5 % ($p <0.05$).

3. Results and Discussion

3.1. Biomass production

The model derived growth curves are shown in Figure 1. Figure 1A shows the effect of glycerol over *Chlorella vulgaris* cultivated in wastewater. In all tested conditions this microalgae showed a typical microbial growth. Significantly higher amounts of biomass have been produced at 25 and 50mM of glycerol (111 and 165 mg L^{-1} d^{-1}, against approximately 50 in the other conditions, $p<0.05$). However, with the addition of the lower concentrations of glycerol (6.12 and 12.5 mM), there were significantly reduced productivities (average 47 mg L^{-1} d^{-1}, compared to the 50 mg L^{-1} d^{-1} without glycerol, $p<0.05$). No linear correlation could be established between biomass productivity and glycerol concentration (R^2 = 0.27, with 5 points). The values of productivity (table 3) are similar to data showed by Liang and coworkers (2009) for growth of *Chlorella vulgaris* with addition of glycerol as organic carbon source. Testing different concentrations of glycerol with *C. vulgaris*, in synthetic medium, it was found an inhibitory effect in concentrations higher than 100mM in media. However, there was 8-fold higher biomass productivity in mixotrophic conditions with 100mM of glycerol, when compared to autotrophic cultivation (102 mg L^{-1} d^{-1} against 13 mg L^{-1} d^{-1}). Chen and Walker (2011) studied *Chlorella protothecoides*, achieving a maximum productivity of 3.9 g L^{-1} d^{-1} in batch systems with synthetic medium. This species of microalgae is known for producing higher amounts of biomass. *Chlorella pyrenoidosa* instead, presented very low productivities values with diluted (10%) treated piggery wastewater in mixotrophic conditions: 30 mg L^{-1} d^{-1} for biomass and 6.3 mg L^{-1} d^{-1} for lipids (Wang *et al.*, 2012). Another algae well studied in mixotrophic conditions is the diatom *Phaeodactylum tricornutum*, which have presented productivity values from 0.5 to 2.3 g L^{-1} d^{-1}, in synthetic media (Ceron-Garcia, 2000;2005;2006; Morais *et al.*, 2009). In the present work *C. vulgaris* presented values of productivity ranging from 52 to 111 mg L^{-1} d^{-1}, the highest at 25 and 50 mM concentrations of glycerol.

The Figure 1B shows the results with the microalgae *Botryococcus terribilis*. This species is not well studied what so ever. Komaréck and Marvan (1992) have described it occurring in natural populations of *Botryococcus*. Recently, Nascimento and coworkers (2011) have studied its productivity and fatty acids profile aiming biodiesel production. In the present

study, *B. terribilis* presented satisfactory growth in all tested concentrations of glycerol in wastewater, achieving from 128 mg L^{-1} d^{-1} to 165 mg L^{-1} d^{-1} of biomass productivity in wastewater and with addition of 25 and 50 mM of glycerol. In the lowest glycerol concentrations, like the *C. vulgaris* strain, the biomass yields were lower (around 55 mg L^{-1} d^{-1}). *B. terribilis* also have not presented acceptable correlation between glycerol concentration and the growth parameters (R^2 = 0.60). However, the productivity showed by this strain is coherent with values presented by other authors for the *Botryococcus* genus in synthetic media. Sawayama and coworkers (1998), studying a strain of *Botryococcus braunii* reported 28 mg L^{-1} d^{-1} as productivity, grown in secondary treatment wastewater. Órpez and coworkers (2009), using similar effluent from secondary treatment found productivity value of 46 mg L^{-1} d^{-1}, significantly higher than the 33 mg L^{-1} d^{-1} resulted in synthetic medium. Still with *B. braunii*, Kalacheva (2005) reported also 28 mg L^{-1} d^{-1} for productivity, while Tanoi and coworkers (2011), testing mixotrophic cultivation with 40 mM of glucose found 19 mg L^{-1} d^{-1}, and 1.6 mg L^{-1} d^{-1} for the autotrophic cultivation. Eroglu and coworkers (2010), studying 6 different strains of *Botryococcus* reported productivities from 80 to 195 mg L^{-1} d^{-1} in synthetic media. Such results indicate the glycerol as a suitable carbon source for increased microalgae biomass production.

3.2. Nutrient removal

Both strains have presented high values of nutrients removal efficiency (in average > 70%, available in table 2). *Chlorella vulgaris* have presented COD removal efficiency higher than 90% in brute wastewater (WW) and not significantly smaller with addition of 6.1 and 12.5 mM of glycerol. Similar results were achieved by *B. terribilis* (table 2). Such findings show that almost all the organic carbon available in the medium was consumed (measured by COD reduction). Microalgae are not primarily efficient in consuming COD from brute wastewater, since in several cases there is a part as organic matter, which demands degradation by bacterial systems (Chan *et al*., 2011). However, both strains have presented excellent results in the present work. It can be explained by the fact that the COD concentration in the brute wastewater (1000,25 mg L^{-1} O_2) was practically the same for filtered samples (1000,12 mg L^{-1} O_2), showing that all COD present was in the soluble form.

The European Directive 98/15/EC establish a COD removal percentage in the wastewater treatment of 75% (or up to 125 mg L^{-1} O_2). *Chlorella vulgaris* removed the COD concentration below legal limits in the wastewater (20 mg L^{-1} O_2), and even in the lower glycerol concentrations of 6.1 and 12.5 mM it has achieved the legal threshold (60 mg L^{-1} O_2 and 121 mg L^{-1} O_2, respectively). Those values of COD removal are higher than the 50% presented by *C. pyrenoidosa*, mixotrophic cultivated in treated piggery wastewater (Wang *et al*., 2012). Other authors have reported COD removal percentages by autotrophic *Chorella* ranging from 50 to 83% (González et al., 2008; Wang et al., 2009). Godos and coworkers (2009) have reported 70 to 90% of COD removal in microalgae consortium in stabilization pounds feed with piggery wastewater. With the higher glycerol concentrations it was not achieved the legal limits, remaining 1012 mg L^{-1} O_2 (25mM) and 1672 mg L^{-1} O_2 (50mM). However, it is worth noticing the high removal percentage (average 73%) presented in those conditions of high initial concentrations.

The nitrogen series showed also high values of removal percentage, over 90% (average consumption of 45 mg L^{-1} as total nitrogen). Several studies highlight the general preference of microalgae for ammonium when available among other nitrogen forms (Richmond, 2004; Park *et al*., 2011). It can be noticed in the remaining amount of nitrate (NO_3) at the end of trials (average 10%), contrasted with the total ammonium consumption (tables 1 and 2). Such removal values attend the European directive 98/15/EC that establishes 10 mg L^{-1} for nitrogen in disposing effluents, in all experimental conditions. The present values of nitrogen removal are higher than the results of Ruiz-Marin and coworkers (2010), which showed only 60% o nitrogen removal by *Chlorella vulgaris* in urban treated wastewater. Wang and coworkers (2009) reported also smaller values of nitrogen removal using *Chlorella* sp., around 50%.

Chlorella vulgaris has consumed all available phosphate in the wastewater (7.7 mg L^{-1}), guaranteeing the European directive 98/15/EC legal limit for disposing wastewater (<1mg L^{-1}). Phosphorus is an important cellular component, usually present in the microalgae biomass as 1% of dry weight (Grobeelar, 2004). Because of that, it is almost never a limiting factor to microalgae growth. Sometimes, because of this same reason phosphorus is not completely removed in microalgae-based systems (Akpar & Muchie, 2010; Powell *et al.*, 2008). Godos and coworkers (2009) have reported values <10% for phosphors removal from

treated piggery effluent. However, other authors presented results similar to the ones from the present work for *Chlorella sp.*: between 75 and 85% (Wang *et al*., 2009), 96% (Feng *et al*., 2011), 100% (Ruiz *et al*., 2011), 60% (Ruiz-Marin *et al*., 2010) and 74 % (Sydney *et al*., 2011).

Botryococcus terribilis has presented COD removal efficiency higher than 90% in brute wastewater (WW) and not significantly smaller with addition of 6.1 and 12.5 mM of glycerol. It guaranteed the attendance to the European directive 98/15/EC, regarding to COD disposal. In the other hand, in the higher glycerol concentrations (25 and 50 mM) there were significantly smaller removal percentages (table 2). However, it is important to highlight the elevated COD concentrations at the beginning of the trials (3700 and 6000mg L^{-1}) and still it was observed a reduction of 73%.

The nitrogen removal was higher than 95% as total nitrogen (table 2). There was no significant difference in the total nitrogen and ammonium uptake by *B. terribilis* ($p>0.05$). There were observed values of removal >93% for total nitrogen, 100% for NH_4 and between 77 – 89% for NO_3. However, the nitrate showed, like *C. vulgaris*, a smaller removal percentage compared to ammonium ($p<0.05$). This highlights the microalgae preference for ammonium as a nitrogen source (Richmond, 2004; Ruiz *et al*., 2011; Park *et al*., 2011). Sawayama and coworkers (1994) reported nitrogen removal percentages of only 25% for B. braunii cultivated in secondary treatment effluent.

Botryococcus terribilis showed a smaller phosphorus uptake in wastewater and with the addition of 6.1mM glycerol. Because of that, the European directive 98/15/EC was no achieved in those conditions, which presented final phosphorus concentrations of 4.3 and 4.5 mg L^{-1} respectively. At higher glycerol concentrations (12.5 to 50 mM) all phosphorus was removed, which may be explained by the enhanced microalgae growth in the availability of the organic carbon (Wang *et al*., 2012; Lee, 2004). Sawayama and coworkers (1994) reported only 51% of phosphorus removal from secondary treatment effluent in *B. braunii* cultivation systems.

3.3. Biochemical composition

Table 4 summarizes the biochemical composition of both strains biomasses in all experimental conditions. *Chlorella vulgaris* presented a correlation between the COD

concentration and the carbohydrates contents (R^2 = 0.80). The opposite result was observed for the *Botryococcus* strain (R^2 = 0.0). However, it has presented higher percentages of carbohydrates than *Chlorella* (table 4). Both strains have presented high protein content, from 70 to 80% average. No correlation could be established between protein content and glycerol concentrations (R^2 = 0.13 for *Chlorella* and R^2 =0.09 for *Botryococcus*).

Regarding to the pigment content, it was observed a weak negative correlation between glycerol concentrations with chlorophyll (R^2 = 0.71 and 0.74, for *Chlorella* and *Botryococcus* respectively) and carotenoids (R^2 = 0.69 and 0.79, for *Chlorella* and *Botryococcus* respectively) production.

The total lipids presented values form 13 to 27% of dry weight for *C. vulgaris*. Those values are similar to data reported for this species by other authors in similar conditions (Bertoldi *et al.*, 2006; González *et al*., 2008; Sydney *et al*., 2011; Voltolina *et al*., 1999). No correlation could be established between glycerol concentration and lipid content (R^2 = 0.61). Nevertheless, higher amounts of lipids are found in brute wastewater, 27%, and with smaller concentrations of glycerol (21 and 24%). With the higher glycerol addition, 25 and 50mM, there was observed a significant reduction of lipid content, to 13 and 15 % ($p<0.05$). Chen and Walker (2011) reported a lipidic productivity of 2.0 g L^{-1} d^{-1} for C*hlorella protothecoides* in mixotrophic cultivation. It represents a 55% of lipids as dry weight. However, it is noticeable that it was provided 30g per liter of crude glycerol along with 4 g per liter of yeast extract.

Botryococcus terribilis presented a relative small lipid content in wastewater of 25% (23 mg L^{-1} d^{-1} lipid productivity), when compared to the outcomes of other *Botryococcus* strains in similar conditions. The same strain has produced 49% of the dry weight as lipids, cultivated in standard conditions (Nascimento *et al.*, 2011). However, Órpez and coworkers (2009), studying *B. braunii* reported a lipid content of only 18% for its cultivation in secondarily treated effluents (1,19 mg L^{-1} d^{-1} lipid productivity). This is a worldwide known species for its ability to produce up to 60% of dry weight as lipids (Metzger, 2005). Such low productivities may be explained by the high values of nutrients available in the media. Microalgae are known to produce more lipids as a response to nutrient deficiency, especially nitrogen (Hu, 2008). Sawayama and coworkers (1998), also using secondary treatment effluent with *B. braunii*, reached 64% of total lipids as dry weight. However, it is important to

highlight the low productivity of their system, since only 18 mg L^{-1} d^{-1} of lipid productivity has been reported.

The glycerol presented no correlation with the lipid productivity (R^2 = 0.19). There was a not statistical increase with 12.5 mM glycerol addition (26% of lipids, $p>0.05$). However, it were observed significantly lower ($p<0.05$) values with 6.1 mM (9.5%), 25 mM (16%) and 50mM (12%). Such results, for both strains, suggest that glycerol addition is not feasible to produce lipids. Further research must be applied to other strains, since there are data pointing to lipid increase under microalgae in mixotrophic cultivation (Xu *et al.*, 2006; Chen & Walker, 2011).

4. Final remarks

Both strains have presented a satisfactory growth in wastewater and with glycerol addition, with biomass productivities ranging from 47 to 111 mg L^{-1} d^{-1} for *Chlorella vulgaris* and from 55 to 165 mg L^{-1} d^{-1} for *Botryococcus terribilis*.

Even tough no linear correlation could be established between glycerol concentrations and biomass production for both strains, it was observed a significant increase of biomass with 25 and 50 mM glycerol addition. This indicates the glycerol as a suitable carbon source for microalgae biomass production.

Both strains have presented high values of nutrients removal efficiency above 70% for COD, N and P, in average. Glycerol enhanced, not significantly, the nutrient uptake. The only exception was for PO_4 removal by *B. terribilis*, which was significantly higher from 12.5 to 50 mM glycerol addition ($p<0.05$). All tested conditions achieved the legal threshold established by European directive 98/15/EC for COD (125 mg L^{-1}), N (10 mg L^{-1}) and P (mg L^{-1}) in wastewater (WW) and with addition of 6.1 and 12.5 mM glycerol. The exception was also *B. terribilis*, in wastewater (WW) and with addition of 6.1 mM glycerol, in which 4.3 and 4.5 mg L^{-1} PO_4 remained, respectively.

Both strains presented expected values of carbohydrates and protein. The pigments have showed negative correlation between glycerol concentrations. The lipid productivity, even tough not correlated with glycerol availability, was significantly decreased with high concentrations (25 and 50nM). Such results suggest that glycerol addition is not recommended to produce lipids from both strains. Further research must be applied to other strains, since there are data pointing to lipid increase in microalgae under mixotrophic cultivation.

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6. References

AKPAR, OB;MUCHIE,M. Bioremediation of polluted wastewater influent: Phosphorus and nitrogen removal. **Scientific Research and Essays**, v.5, n. 21, p. 3222-3230, 2010.

ANP - National Agency for Oil, Natural Gas and Biofuels. Statistical data. Available in: <http://www.anp.gov.br >. 2010.2

APHA. Standard Methods for the Examination of Water and Wastewater. **American Public Health Association,** Washington, DC. 1995

BENERJEE, A *et al*. *Botryococcus* braunii: a renewable source of hydrocarbons and other chemicals. **Critical Reviews in Biotechnology**, v. 22, n.3, p. 245-279, 2002.

BENNEMAN,JR; MIYAMOTO, K; HALLEBECK, PC. Bioengineering aspects of biophotolysis. **Enzyme and Microbial Technology**, v. 2, p.103–111, 1980.

BERTOLDI, FC *et al*. Lipids, fatty acids composition and carotenoids of Chlorella vulgaris cultivated in hydroponic wastewater. **Grasas y Aceites**, v. 57, n.3, p. 270-274, 2006.

CAREY, RO; MIGLIACCIO, KW. Contribution of Wastewater Treatment Plant Effluents to Nutrient Dynamics in Aquatic Systems: A Review. **Environmental Management**, v. 44, p. 205–217, 2011.

CERÓN GARCIA, MC *et al*. Mixotrophic growth of Phaeodactylum tricornutum on glycerol: growth rate and fatty acid profile. **J. of Applied Phycology**. v. 12, p. 239-248, 2000.

CERÓN GARCIA, MC *et al*. Mixotrophic growth of the microalga Phaeodactylum tricornutum Influence of different nitrogen and organic carbon sources on productivity and biomass composition. **J. Process Biochemistry**. v.40, p. 297-305, 2005.

CERÓN GARCIA, MC *et al*. Mixotrophic production of marine microalga Phaeodactylum tricornutum on various carbon sources**. J. Microbiol. Biotechnol** v. 16, n.5, p. 689-694, 2006.

CHAN, YJ *et al*. A review on anaerobic-aerobic treatment of industrial and municipal wastewater. **Chemical Engineering Journal**, v. 155, n. 1-2, p. 1-18, 2011.

CHEN, Y;WALKER, TH. Biomass and lipid production of heterotrophic microlgae Chlorella protothecoides by using biodiesel-derived crude glycerol. **Biotechnology letters**, v. 33, p. 1973-1983, 2011.

CHISTI, Y. Biodiesel from Microalgae. **Biotechnology Advances**, v.25, p**.** 294-306,2007.

DAYANANDA, C *et al*. Autotrophic cultivation of *Botryococcus* braunii for the production of hydrocarbons and exoplysaccharides in various media. **Biomass and Bioenergy**, v. 31, p. 87-93, 2007.

EROGLU,E;OKADA,S;MELIS,A. Hydrocarbon productivities in different *Botryococcus* strains: comparative methods in product quantification. **J Appl Phycol**, v. 23, n. 4, p. 763-775, 2010.

GODOS, I *et al*. Long-term operation of high rate algal ponds for the bioremediation of piggery wastewaters at high loading rates. **Bioresource Technology**, v. 100, p. 4332–4339, 2009.

GONZÁLEZ, C *et al*. Efficient nutrient removal from swine manure in a tubular biofilm photobioreactor using algae-bacteria consortia. **Water Science and Technology**, v. 59, n. 1, p. 95 - 102, 2008.

GROBELAAR,JU. Algal Nutrition: Mineral Nutrition. In: Richmond, A (ed). **Handbook of microalgal culture: biotechnology and applied phycology**. Blackwell Publishing, 1 Ed., p. 97-115, 2004.

HAAS, D; TUNNER, W. Regulation of Hexose Transport in Chlorella vulgaris. **PlantPhysiol**, v. 53, p. 14-20, 1974.

HU, Q. *et al*., Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. **The plant Journal**, n. 54, 621-639, 2008

JOHNSON,TD; TACONI, KA. The Glycerin Glut: Options for the Value-Added Conversion of Crude Glycerol Resulting from Biodiesel Production. **Environmental Progress**, v. 26, n. 4, p. 338-348, 2007.

KALACHEVA,GS. The Effect of Temperature on the Lipid Composition of the Green Alga *Botryococcus*. **Microbiology**, v. 71, n. 3, p. 286–293, 2005.

KOMARÉCK, J; MARVAN, P. Morphological Differences in Natural Populations of the Genus *Botryococcus* (Chlorophyceae). **Archiv für Protistenkunde**, v. 141, n. 1-2, p. 65 -100, 1992.

LAM, MK; LEE, KT. Microalgae biofuels: a critical review of issues, problems and the way forward. **Biotechnology Advances**, in press, 2011.

LARSDOTTER, K. Wastewater treatment with microalgae – a literature review. **VATTEN**, v. 62, p. 31–38, 2006.

LEE, Y. Algal Nutrition: Heterotrophic Carbon Nutrition. In: Richmond, A (ed). **Handbook of microalgal culture: biotechnology and applied phycology**. Blackwell Publishing, 1 Ed., p. 116- 124, 2004.

LIANG, Y.; SARKANY, N.; Cui ,Y. Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrphic growth conditions. **Biotechnol Lett** ,v.31,p.1043-1049,2009.

MALARKEY, B; ADDUCCI, J. Water industry review: M&A market outlook 2011. **Boenning & Scattergood Report**. 17 p. 2011.

METZGER, Pierre; LARGEAU, Claude. *Botryococcus* braunii a rich source for hydrocarbons and related esther lipids. **Applied Microbiology and Biotechnology**, v. 66 p. 486-496, 2005.

MORAIS *et al*. PHAEODACTYLUM TRICORNUTUM MICROALGAE GROWTH RATE IN HETEROTROPHIC AND MIXOTROPHIC CONDITIONS Engenh**aria Térmica (Thermal Engineering),** v. 8, n 01, p 84-89, 2009.

NASCIMENTO, IA *et al*. Screening microalgae strains for biodiesel production: lipid productivity and estimation of fuel quality based on fatty-acids profiles as selective criteria. **Bioenergy Research**, in press, 2011.

ORGANIZATION FOR ECONOMIC COOPERATION AND DEVELOPMENT; FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS(OECD/ FAO). Agricultural Outlook 2009-2018.2009 Report. 95 p. Disponível em: < http://pt.scribd.com/doc/58616471/OECD-Agri-Outlook-09-18> . Acesso em: 10 ago. 2011.

ÓRPEZ, R. et al. Growth of the microalga *Botryococcus* braunii in secondarily treated sewage. **Desalination**, v. 246, p. 625 – 630, 2009.

OSWALD, WJ; GOTTAS, HB. Photosynthesis in sewage treatment. **American Society of Civil Engeneering**, n. 2849, p. 73-105, 1957.

PARK, JBK;CRAGGS, RJ; SHILTON, AN. Wastewater treatment high rate algal ponds for biofuel production. **Bioresource Technology,** v. 102, p. 35-42, 2011.

PÉREZ-GARCIA, O *et al*. Efficiency of growth and nutrient uptake from wastewater by heterotrophic, autotrophic, autotrophic and mixotrophic cultivation of Chlorella vulgaris immobilized with azospirillum brasiliense. **J. Phycol**, v. 46, p. 800–812, 2010.

POWELL *et al*. Factors Influencing Luxury Uptake of Phosphorus by Microalgae in Waste Stabilization Ponds. **Environ. Sci. Technol**, v. 42, p. 5958–5962, 2008

RANGA RAO, A.; SARADA, R.; RAVISHANKAR G.A. Influence of CO2 on growth and hydrocarbon production in *Botryococcus* braunii. **Journal Microbiol Biotechnol**,n.3,p.414-9, Mar.17,2007.

RAWAT, I *et al*. Dual role of microalgae: phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. **Applied Energy**, v. 88, p. 3411 – 3424, 2011.

RUIZ *et al*. Effect of Nitrogen and Phosphorus Concentration on Their Removal Kinetic in Treated Urban Wastewater by Chlorella Vulgaris. **International Journal of Phytoremediation**, v. 13, p. 884–896, 2011.

RUIZ-MARIN,A;MENDOZA-ESPINOSA, LG; STEPHENSON, T. Growth and nutrient removal in free and immobilized green algae in batch and semi-continuos cultures treating real wastewater. **Bioresource Technology,** v. 101, p. 58-64, 2010.

SAWAYAMA, S;INOUE, S; YOKOYAMA, S. Continuous culture of hydrocarbon-rich microalga *Botryococcus* braunii in secondarily treated sewage. **Applied Microbiology Biotechnology**, v. 41, p. 729-731, 1994.

SCHUCHARDT, U; SERCHELI, R; VARGAS, R.M. Transesterification of vegetable oils: a review. **Journal of Brazilian Chemical Society,** v. 9, p. 199-210, 1998.

SYDNEY *et al.* Screening of microalgae with potential for biodiesel production and nutrient removal from treated domestic sewage. **Applied Energy**, v. 88, p. 3291-3294, 2011.

TANOI, T *et al*. Effects of carbon source on growth and morphology of *Botryococcus* braunii. **Journal of Applied Phycology,** v. 23, p. 25-33, 2011**.**

UNO. The United Nations World Water Development Report 3. WATER IN A CHANGING WORLD. 2009

VOLTOLINA D, *et al*. Growth of Scenedesmus sp. in artificial wastewater. **Bioresource Technology**, v. 68, p. 265–268, 1999.

WANG, L *et al*. Cultivation of green algae Chlorella sp. in different wastewaters from municipal wastewater treatment plant. **Applied Biochemistry and Biotechnology**, v. 162, n. 4, p 1174-1186, 2010.

XU *et al.* High quality biodiesel production from a microalga Chlorella protothecoides by heterotrophic growth in fermenters*.* **Journal of Biotechnology**, v. 126, n. 4, p. 499-507, 2006.

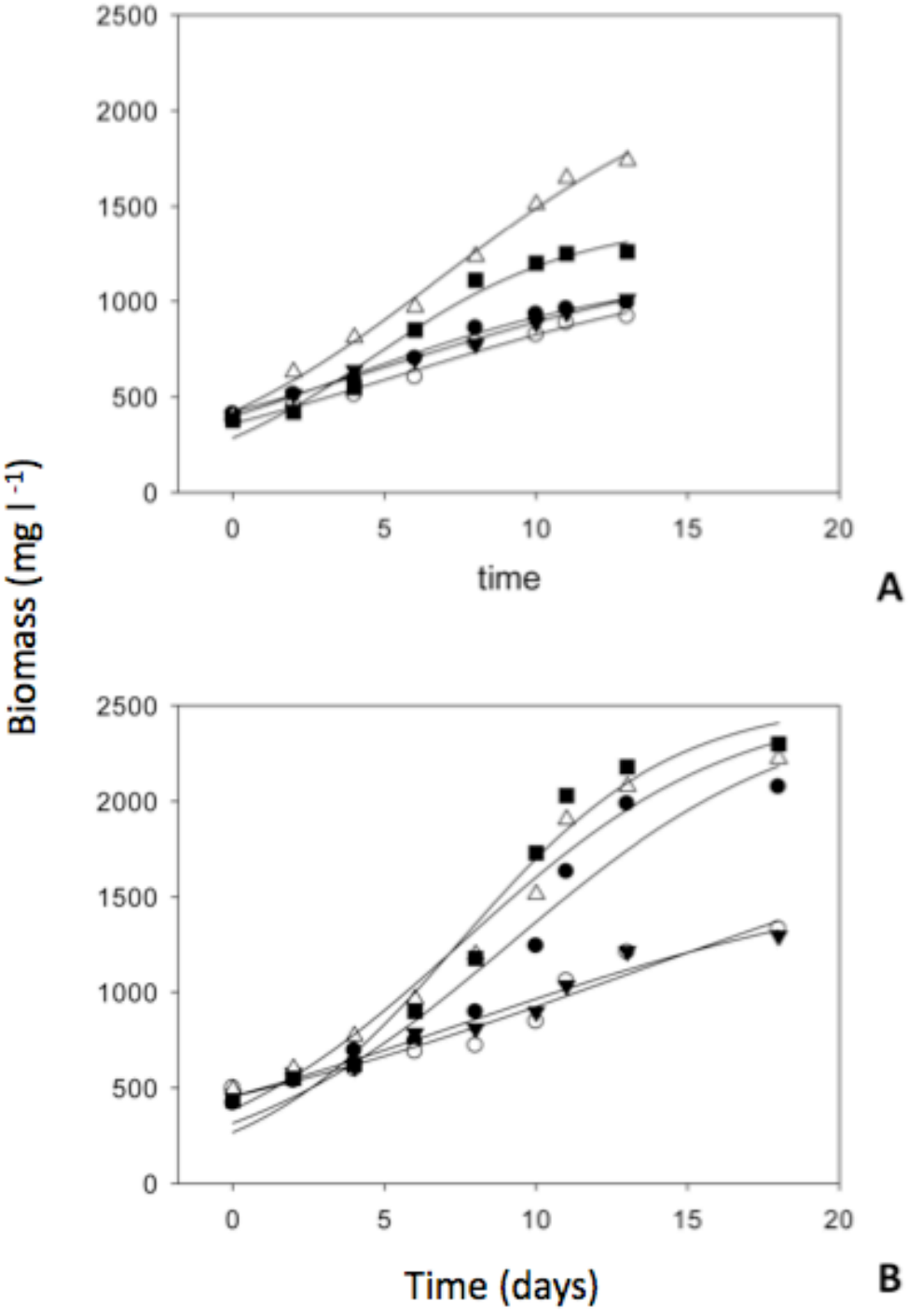

Figure 1: Growth curves of the strains in all tested conditions: *Chlorella vulgaris* in wastewater (WW) ▼, with glycerol addition of 6.1 mM○, 12.5 mM ●, 25mM△ and 50 mM■ (A) and *Botryococcus terribilis* in wastewater (WW) ●, with glycerol addition of 6.1 mM○, 12.5 mM ▼, 25mM△ and 50 mM■ (B).

Table 1: Chemical composition of brute wastewater (WW) and in all experimental conditions for both strains at the beginning of the trials.

		Chlorella vulgaris					*Botryococcus terribilis*				
Parameters	Brute WW	WW	WW +6.1 mM	WW+12.5 mM	WW+25 mM	WW +50 mM	WW	WW +6.1 mM	WW+12.5 mM	WW+25 mM	WW +50 mM
Turbidity (NTU)	280	265	225	260	260	220	205	240	215	200	195
Salinity ($‰_o$)	0	-	-	-	-	-	-	-	-	-	-
Viscosity (µ.Kg.m.s)	0,023	0,021	0,017	0,021	0,019	0,025	0,02	0,021	0,02	0,021	0,023
pH	6,91	7,19	7,205	7,2	7,165	7,165	7,245	7,205	7,205	7,14	7,185
COND (µS/cm)	808	-	-	-	-	-	-	-	-	-	-
SS (g L^{-1})	0,26	0,41	0,38	0,4	0,4	0,38	0,42	0,5	0,46	0,49	0,43
COD (mg O_2L^{-1})	1000,1	1002,3	1012,9	2025,9	3750,8	6195,6	995,63	1095,11	2190,22	3850	6078,9
Nt (mgN L^{-1})	52,03	43,67	48,63	47,56	49,54	46,78	51,23	52,41	50,39	48,86	50,02
NH_4 (mgN-NH_4 L^{-1})	43,85	39,44	44,02	43,85	44,19	42,83	47,42	46,06	45,89	44,53	45,72
NO_3 (mgN-NO_3 L^{-1})	3,06	2,47	1,91	2,06	1,93	2,2	3,18	2,41	2,01	2,56	2,64
NO_2 (mgN-NO_2 L^{-1})	0,13	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
PO_4 (mgP-PO_4 L^{-1})	7,11	6,66	9,32	9,17	9,37	9,79	11,50	11,41	10,84	11,59	10,56

Table 2: Nutrient removal efficiency (%) of both *C. vulgaris* and *B. terribilis* strains in all tested conditions.

	Chlorella vulgaris					*Botryococcus terribilis*				
Parameters	WW	WW +6.1 mM	WW+12.5 mM	WW+25 mM	WW +50 mM	WW	WW +6.1 mM	WW+12.5 mM	WW+25 mM	WW +50 mM
COD (%)	98a	94,1a	94,2a	73,7b	73,0b	97,1a	95a	95,5a	79,6b	83,5c
NT (%)	95,5a	94,1a	92,4a	94,8a	95a	96,4a	94,2a	93,6a	94,6a	95a
NH_4 (%)	99,9a	99,9a	99,9a	99,9a	99,9a	99,9a	99,9a	99,8a	99,9a	99,9a
NO_3 (%)	91,7a	89,3a	90,1a	89,4a	90,7a	89,7a	77b	82,6c	86,1a	82,8c
PO_4 (%)	100a	100a	100a	100a	100a	62,2a	59,9a	100b	100b	100b

*Different letters show statistical significance ($p< 0.05$) by ANOVA test. Test carried out to compare the parameters among the treatments within each strain.

Table 3: Verlhust kinetics parameters of both strains in all tested conditions (model fitted using STATISTICA software with $R^2 > 0.80$ and p-values < 0.05). Lipid productivity (LP) was determined according with Grifiths (2009), following the equation *LP = Lipid content (mg)˙ Productivity*.

	Chlorella vulgaris					*Botryococcus terribilis*				
Parameters	WW	WW +6.1 mM	WW+12.5 mM	WW+25 mM	WW +50 mM	WW	WW +6.1 mM	WW+12.5 mM	WW+25 mM	WW +50 mM
X_O (mg SS·L^{-1})	0,410	0,400	0,420	0,421	0,380	0,420	0,460	0,490	0,450	0,460
X_M (mg SS·L^{-1})	1,013a	0,94b	1,008a	1,775c	1,311d	2,17a	1,39b	1,31b	2,28c	2,37c
μ (day^{-1})	0,08	0,08	0,08	0,13	0,11	0,12	0,07	0,07	0,11	0,13
Productivity (mg SS·L^{-1}·d^{-1})	52,26a	47,75b	47,08b	111,59c	89,71d	128,95a	57,53b	55,48b	140,21c	165,79d
R^2	0,995	0,988	0,996	0,996	0,981	0,944	0,940	0,969	0,981	0,977
p –value	0,001	0,009	0,001	0,000	0,005	0,003	0,003	0,013	0,000	0,000
Culture days	13	13	13	13	13	18	18	18	18	18
Lipid productivity (mg·L^{-1}·d^{-1})	14,25a	10,39b	11,43b	14,95a	14,11a	32,24a	5,49b	14,42c	23,34d	20,56e

*Different letters show statistical significance ($p< 0.05$) by ANOVA test. Test carried out to compare the parameters Xm, μ and productivity among the treatments within each strain.

Table 4: Biochemical composition of the microalgae strains as percentage of dry weight (%) and concentration in dry weight (µg/mg).

Strain/ condition	Biomass composition				
C. vulgaris	Carbohydrates (%)	Total lipids (%)	Protein (%)	Chlorophyl α (µg/mg)	Total carotenoids (µg/mg)
WW	3,9a	27,3a	65,0a	4,71a	1,81a
WW + 6.12 mM	5,2ab	21,7b	75,0b	2,53b	0,86b
WW + 12.5 mM	5,9ab	24,3a	69,0c	2,25b	0,84b
WW + 25 mM	6,8b	13,4c	79,0d	0,20c	0,04c
WW + 50 mM	7,3b	15,7c	72,0e	0,25c	0,04C
B. terribilis					
WW	7,8a	25,0a	68,0a	3,76a	0,84a
WW + 6.12 mM	7,1ab	9,5b	83,0b	2,66b	0,74b
WW + 12.5 mM	6,2ab	26,0a	70,0a	1,38c	0,52c
WW + 25 mM	4,9b	16,7c	75,0c	0,36d	0,05d
WW + 50 mM	7,9a	12,4d	78,0d	0,19e	0,00d

* Different letters show statistical significance ($p < 0.05$) by ANOVA test. Test carried out to compare each biochemical fraction, among the treatments within each strain.

5. CONSIDERAÇÕES FINAIS

A microalga *Chorella vulgaris* apresentou crescimento satisfatório em diferentes efluentes domésticos tratados provenientes de estações de tratamento (ETE) da Espanha, alcançando produtividades entre 50 e 150 mg L^{-1} d^{-1}. Tais resultados apontam para a viabilidade de efluentes domésticos como fonte de nutrientes para o cultivo de microalgas;

Em todas as amostras foi observada a remoção de nutrientes (medidos em nitrogênio, fósforo e demanda química de oxigênio). A maioria apresentou concentrações abaixo dos limites legais: <10 mg L^{-1} para nitrogênio, <1 mg L^{-1} para fósforo e <125 mg L^{-1} O_2 para demanda química de oxigênio.

A suplementação de CO_2 é uma importante variável para a produção de biomass e eficiente remoção de nutrientes. *Chlorella vulgaris* foi capaz de fixar em biomassa entre 56 e 242 mg por litro por dia de CO_2.

Modificações artificiais da razão N/P não aumentou a produção de biomassa nem a taxa de remoção de nutrientes, para os cultivos com efluente da secagem dos lodos (centrate). Tais resultados reinforçam a aplicabilidade de efluentes urbanos tratados como fonte de nutrientes para o cultivo de microalgas.

Nos experimentos com efluente doméstico, proveniente de uma ETE do Estado da Bahia/Brasil suplementado com glicerina, foi observado crescimento satisfatório das microalgas *Chlorella vulgaris* e *Botryococcus terribilis.* As cepas apresentaram produtividades entre 47 - 111 mg L^{-1} d^{-1} e entre 55 - 165 mg L^{-1} d^{-1}, respectivamente.

Embora não tenha sido possível estabelecer correlação linear entre as concentrações de glicerina e a produção de biomassa, foi observado aumento significativo de biomassa com

25 e 50 mM de glicerina. Tais resultados indicam a possibilidade de aplicar a glicerina como fonte de carbono orgânico para a produção de biomassa microalgal. Também foi possível observar altos valores de remoção de nutrientes, acima de 70% para DQO, nitrogênio e fósforo. A glicerina pode aumentar a taxa de remoção de nutrientes em algumas condições.

Ambas *Chlorella vulgaris* e *Botryococcus terribilis* apresentaram valores esperados de carboidratos e proteínas. Os pigmentos fotossintéticos apresentaram correlação negativa com as concentrações de glicerina. As produtividades lipídicas, embora não correlacionadas com a disponibilidade de glicerina, foram reduzidas significativamente nas menores concentrações de 6.1 e 12.5 mM (de 14 para 10 mg L^{-1} d^{-1}), e permaneceram significativamente iguais nas maiores concentrações, 25 e 50 mM, para *Chlorella vulgaris*. Para *B. terribilis* as produtividades foram extremamente reduzidas nas menores concentrações (de 32 para 5 e 14 mg L^{-1} d^{-1}) e nas maiores também (de 32 para 20 mg L^{-1} d^{-1}).

6. REFERÊNCIAS

AKPAR, OB;MUCHIE,M. Bioremediation of polluted wastewater influent: Phosphorus and nitrogen removal. **Scientific Research and Essays**, v.5, n. 21, p. 3222-3230, 2010.

ALTIERI, Miguel A. The ecological impacts of large-scale agro fuel monocultures production systems in the Americas**. Bulletin of Science, Technology & Society**, v. 29, n. 3, p. 236-244.2009.

APHA. Standard Methods for the Examination of Water and Wastewater. **American Public Health Association,** Washington, DC. 1995

BAUMGARTEN, E; NAGEL, M; TISCHNER, R. Reduction of the nitrogen and carbon content in swine waste with algae and bacteria. **Appl Microbiol Biotechnol**, v. 52, p. 281-284, 1999.

BENNEMAN,JR; MIYAMOTO, K; HALLEBECK, PC. Bioengineering aspects of biophotolysis. **Enzyme and Microbial Technology**, v. 2, p.103–111, 1980.

BERTOLDI, FC *et al*. Lipids, fatty acids composition and carotenoids of Chlorella vulgaris cultivated in hydroponic wastewater. **Grasas y Aceites**, v. 57, n.3, p. 270-274, 2006.

BHATNAGAR, A *et al*. Chlorella minutissima – a promising fuel alga for cultivation in municipal wastewater. **Applied Biochemical Biotechnology**, v. 161, p. 523-536, 2010.

BHATNAGAR, A *et al*. Renewable biomass production by mixotrophic algae in the presence of various carbon sources and wastewaters. **Applied Energy**, v. 88, p. 3425–3431, 2011.

BROWN, L. M.; ZEILER, K. G. Aquatic biomass and carbon dioxide trapping. **Energy Conversion and Management,** v. 34, p.1005-10013, 1993.

CAREY, RO; MIGLIACCIO, KW. Contribution of Wastewater Treatment Plant Effluents to Nutrient Dynamics in Aquatic Systems: A Review. **Environmental Management**, v. 44, p. 205–217, 2011.

CERÓN GARCIA, MC *et al*. Mixotrophic growth of Phaeodactylum tricornutum on glycerol: growth rate and fatty acid profile. **J. of Applied Phycology**. v. 12, p. 239-248, 2000.

CERÓN GARCIA, MC *et al*. Mixotrophic growth of the microalga Phaeodactylum tricornutum Influence of different nitrogen and organic carbon sources on productivity and biomass composition. **J. Process Biochemistry**. v.40, p. 297-305, 2005.

CERÓN GARCIA, MC *et al*. Mixotrophic production of marine microalga Phaeodactylum tricornutum on various carbon sources**. J. Microbiol. Biotechnol** v. 16, n.5, p. 689-694, 2006.

CHAN, YJ *et al*. A review on anaerobic-aerobic treatment of industrial and municipal wastewater. **Chemical Engineering Journal**, v. 155, n. 1-2, p. 1-18, 2011.

CHEN, Y;WALKER, TH. Biomass and lipid production of heterotrophic microlgae Chlorella protothecoides by using biodiesel-derived crude glycerol. **Biotechnology letters**, v. 33, p. 1973-1983, 2011.

CHISTI, Y. Biodiesel from Microalgae. **Biotechnology Advances**, v.25, p**.** 294-306,2007.

DE PAUW, N. & VAERENBERGH, E. Microalgal wastewater treatment systems: potentials and limits. In P. F. Ghetti (ed.), **Phytodepuration and the employment of the biomass produced.** Itália, Reggio Emilia, p. 211-287, 1983.

DUNAHAY, TG *et al*. Manipulation of microalgal lipid production using genetic engineering. **Appl. Biochem. Biotechnol**, v. 57, p. 8223 – 8231, 1996.

EROGLU,E;OKADA,S;MELIS,A. Hydrocarbon productivities in different Botryococcus strains: comparative methods in product quantification. **J Appl Phycol**, v. 23, n. 4, p. 763-775, 2010.

FENG, Y; CHAO, L; ZHANG, D. Lipid production of Chlorella vulgaris cultured in artificial wastewater medium. **Bioresource Technology**, v. 102, p. 101-105, 2011.

FLYNN *et al*. Selection for fitness at the individual or population levels: modelling effects of genetic modifications in microalgae on productivity and environmental safety. **Journal of Theoretical Biology**, v. 263, p. 269-280, 2010.

GODOS, I *et al*. Long-term operation of high rate algal ponds for the bioremediation of piggery wastewaters at high loading rates. **Bioresource Technology**, v. 100, p. 4332–4339, 2009.

GONZÁLEZ, C *et al*. Efficient nutrient removal from swine manure in a tubular biofilm photo-bioreactor using algae-bacteria consortia. **Water Science and Technology**, v. 59, n. 1, p. 95 - 102, 2008.

GONZÁLEZ, LE;CANIZARES,RO;BAENA,S. Efficiency of amônia and phosphorus removal from a colombian agroindustrial wastewater by the microalgae Chlorella vulgaris and Scenedesmus dimorphus. **Bioresource Technology**, v. 60, p. 259-262, 1997.

GRIFFITHS M.J.; HARRISON, S.T.L. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. **Journal of Applied Phycology**, v. 21, n.5,p. 493-507,2009.

GROBELAAR,JU. Algal Nutrition: Mineral Nutrition. In: Richmond, A (ed). **Handbook of microalgal culture: biotechnology and applied phycology**. Blackwell Publishing, 1 Ed., p. 97-115, 2004.

HAAS, D; TUNNER, W. Regulation of Hexose Transport in Chlorella vulgaris. **PlantPhysiol**, v. 53, p. 14-20, 1974.

HU, Q. *et al*., Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. **The plant Journal**, n. 54, 621-639, 2008

INTERGOVERNMENTAL PANEL OF CLIMATE CHANGE. WMO/UNEP/IPCC. **Fourth Assessment Report (AR4 Synthesis Report).** [S.l.]: 52 p, IPCC 2007.

INTERNATIONAL ENERGY AGENCY (IEA, França). Carbon dioxide utilization: evaluation of specific biological processes which have the capability of directly utilizing high concentrations of carbon dioxide as found in a flue gas streams from power generation plant. Report 2008. Disponível em: <http://www.iea.org/textbase/nppdf/free/ 2008/CCS_2008.pdf >. Acesso em: 2 ago. 2011.

JOHNSON,TD; TACONI, KA. The Glycerin Glut: Options for the Value-Added Conversion of Crude Glycerol Resulting from Biodiesel Production. **Environmental Progress**, v. 26, n. 4, p. 338-348, 2007.

KALACHEVA,GS. The Effect of Temperature on the Lipid Composition of the Green Alga Botryococcus. **Microbiology**, v. 71, n. 3, p. 286–293, 2005.

KARGI, F; UYGUR, A. Nutrient removal performance of a sequencing batch reactor as a funtion of the sludge age. **Enzyme and Microbial Technology**, v. 31, p. 842-847, 2002.

KILHAM, SS *et al.* COMBO: a defined freshwater culture medium for algae and zooplankton*.* **Hydrobiologia***,* v. 377, p. 147–159, 1998.

KOMARÉCK, J; MARVAN, P. Morphological Differences in Natural Populations of the Genus Botryococcus (Chlorophyceae). **Archiv für Protistenkunde**, v. 141, n. 1-2, p. 65 -100, 1992.

KONG, Q *et al*. Culture of microalgae *Chlamydomonas reinhardtii* in wastewater for biomass feedstock production. **Applied Biochemistry and Biotechnology**, v. 160, p 9-18, 2010.

KUMAR, MS et al. Influence of nutrient loads, feeding frequency and inoculum source on growth of *Chlorella vulgaris* in digested piggery effluent culture medium. **Bioresource Technology**, v. 101, p. 6012–6018, 2011.

LAM, MK; LEE, KT. Microalgae biofuels: a critical review of issues, problems and the way forward. **Biotechnology Advances**, in press, 2011.

LARSDOTTER, K. Wastewater treatment with microalgae – a literature review. **VATTEN**, v. 62, p. 31–38, 2006.

LEE, Y. Algal Nutrition: Heterotrophic Carbon Nutrition. In: Richmond, A (ed). **Handbook of microalgal culture: biotechnology and applied phycology**. Blackwell Publishing, 1 Ed., p.

116- 124, 2004.

LI, Y *et al*. Integration of algae cultivation as biodiesel production feedstock with municipal wastewater treatment: Strains screening and significance evaluation of environmental factors. **Bioresource Technology,** v**.**102, p. 10861 - 10867,2011.

LIANG, Y.; SARKANY, N.; Cui ,Y. Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrphic growth conditions. **Biotechnol Lett** ,v.31,p.1043-1049,2009.

MALARKEY, B; ADDUCCI, J. Water industry review: M&A market outlook 2011. **Boenning & Scattergood Report**. 17 p. 2011.

METZGER, Pierre; LARGEAU, Claude. Botryococcus braunii a rich source for hydrocarbons and related esther lipids. **Applied Microbiology and Biotechnology**, v. 66 p. 486-496, 2005.

MORAIS *et al*. PHAEODACTYLUM TRICORNUTUM MICROALGAE GROWTH RATE IN HETEROTROPHIC AND MIXOTROPHIC CONDITIONS Engenh**aria Térmica (Thermal Engineering),** v. 8, n 01, p 84-89, 2009.

MUSSGNUG *et al.* Engineering photosynthetic light capture: impacts on improved solar energy to biomass conversion. **Plant Biotechnology Journal**. v. 5, n. 6, p. 802-814, 2007.

NASCIMENTO, IA *et al*. Screening microalgae strains for biodiesel production: lipid productivity and estimation of fuel quality based on fatty-acids profiles as selective criteria. **Bioenergy Resource**, in press, 2011.

NODARI, RO; GUERRA, MP. Riscos de organismos geneticamente modificados. **Cadernos de Ciência & Tecnologia,** v. 18, n. 1, p. 81-116, 2001.

ORGANIZATION FOR ECONOMIC COOPERATION AND DEVELOPMENT; FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS(OECD/ FAO). Agricultural Outlook 2009-2018.2009 Report. 95 p. Disponível em: < http://pt.scribd.com/doc/58616471/OECD-Agri-Outlook-09-18> . Acesso em: 10 ago. 2011.

OSWALD, WJ; GOTTAS, HB. Photosynthesis in sewage treatment. **American Society of Civil Engeneering**, n. 2849, p. 73-105, 1957.

PARK, JBK;CRAGGS, RJ; SHILTON, AN. Wastewater treatment high rate algal ponds for biofuel production. **Bioresource Technology,** v. 102, p. 35-42, 2011.

PÉREZ-GARCIA, O *et al*. Efficiency of growth and nutrient uptake from wastewater by heterotrophic, autotrophic, autotrophic and mixotrophic cultivation of Chlorella vulgaris immobilized with azospirillum brasiliense. **J. Phycol**, v. 46, p. 800–812, 2010.

POWELL *et al*. Factors Influencing Luxury Uptake of Phosphorus by Microalgae in Waste Stabilization Ponds. **Environ. Sci. Technol**, v. 42, p. 5958–5962, 2008

RANGA RAO, A.; SARADA, R.; RAVISHANKAR G.A. Influence of CO2 on growth and hydrocarbon production in Botryococcus braunii. **Journal Microbiol Biotechnol**,n.3,p.414-9, Mar.17,2007.

RAWAT, I *et al*. Dual role of microalgae: phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. **Applied Energy**, v. 88, p. 3411 – 3424, 2011.

ROSENBERG, JN *et al*. A green light for engineered Algae: redirecting metabolism to fuel a biotechnology revolution. **Current Opinion on Biotechnology**, v. 19, n. 5, p. 430-436, 2008.

RUIZ *et al*. Effect of Nitrogen and Phosphorus Concentration on Their Removal Kinetic in Treated Urban Wastewater by Chlorella Vulgaris. **International Journal of Phytoremediation**, v. 13, p. 884–896, 2011.

RUIZ-MARIN,A;MENDOZA-ESPINOSA, LG; STEPHENSON, T. Growth and nutrient removal in free and immobilized green algae in batch and semi-continuos cultures treating real wastewater. **Bioresource Technology,** v. 101, p. 58-64, 2010.

SAWAYAMA, S;INOUE, S; YOKOYAMA, S. Continuous culture of hydrocarbon-rich microalga Botryococcus braunii in secondarily treated sewage. **Applied Microbiology Biotechnology**, v. 41, p. 729-731, 1994.

SCHENK, P. M. *et al*. Second generation biofuels: high efficiency microalgae for biodiesel production**. Bioenergy Research**, v.1,p.20-43,2008.

SCHUCHARDT, U; SERCHELI, R; VARGAS, R.M. Transesterification of vegetable oils: a review. **Journal of Brazilian Chemical Society,** v. 9, p. 199-210, 1998.

SPOLAORE, P., JOANNIS-CASSAN, C., DURAN, E., ISAMBERT, A. 2006. Commercial applications of microalgae**. Journal of Bioscience and Bioengineering**, v. 101, p.87-96, 2006.

STERN, N. ***The economics of climate change*;** the Stern Review. London: Cambridge University Press, Cambridge, 2006. Disponível em: <http://www.fnu.zmaw.de/fileadmin/fnu-files/reports/sternreview.pdf>. Acesso em: 8 ago. 2011.

SYDNEY *et al.* Screening of microalgae with potential for biodiesel production and nutrient removal from treated domestic sewage. **Applied Energy**, v. 88, p. 3291-3294, 2011.

TANOI, T et al. Effects of carbon source on growth and morphology of Botryococcus braunii. **Journal of Applied Phycology**, v. 23, p. 25-33, 2011.

TEIXEIRA, CM & MORALES, ME. Microalga como matéria-prima para a produção de biodiesel. ***Anais do I Congresso da Rede Brasileira de Tecnologia do Biodiesel***. p. 91-96, 2006.

VOLTOLINA D, *et al*. Growth of Scenedesmus sp. in artificial wastewater. **Bioresource Technology**, v. 68, p. 265–268, 1999.

WALTER, TL *et al*. Microalgae as bioreactor. **Plant.Cell Rep**., 24, p.629-641, 2005.

WANG, L *et al*. Cultivation of green algae Chlorella sp. in different wastewaters from municipal wastewater treatment plant. **Applied Biochemistry and Biotechnology**, v. 162, n. 4, p 1174-1186, 2010.

XU *et al.* High quality biodiesel production from a microalga Chlorella protothecoides by heterotrophic growth in fermenters*.* **Journal of Biotechnology**, v. 126, n. 4, p. 499-507, 2006.

ZAH, R. Energy and raw materials—the contributions of chemistry and biochemistry in the future: biofuels—which one is the most ecological one? **Chimia** , v. 61, p. 571–572, 2007.

ZEMKE,P. E; WOOD, B. D.; DYE, D. J. Considerations for the maximum production rates of triacylglycerol from microalgae. **Biomass and Bioenergy** ,v.34 ,p.145-151,2010.