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Composição bioquímica de organismos planctônicos visando à aplicação em aquicultura

Giseli Swerts Rocha

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Tese apresentada ao Programa de Pós-Graduação em Ecologia e Recursos Naturais da Universidade Federal de São Carlos, como parte dos requisitos para a obtenção do título de Doutor em Ciências, área de concentração em Ecologia e Recursos Naturais.

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GISELI SWERTS ROCHA

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Lista de abreviações

ω3 = ômega 3
ANOVA = análise de variância
ABS = absorbância
ALC = álcool alifático livre
AMPL = lipídios polares móveis em acetona
DHA = Ácido docosa-hexanoico
DMSO = Dimetilsulfóxido
DPH = dias após eclosão
EPA = ácido eicosapentanoico
FA = ácidos graxos
FAA = amino ácidos livres
FAMEs = ácidos graxos metil éster
FFA = ácidos graxos livres
FID = detector por ionização em chama
GC = cromatógrafo gasoso
HC = hidrocarbonetos alifáticos
HUFA = ácidos graxos altamente poliinsaturados
KET = cetona
MUFA = ácidos graxos monoinsaturados
PL = fosfolipídios
PUFA = ácidos graxos poliinsaturados
SAFA = ácidos graxos saturados
ST = esterol
TAA = amino ácidos totais
TAG = triacilglicerol
WE/SE = éster de cera

Apresentação do trabalho

Este estudo teve como objetivo principal a avaliação da composição bioquímica (proteínas, carboidratos e lipídios) de organismos aquáticos de três níveis tróficos em várias condições de cultivo. Os organismos utilizados foram a microalga Chlorophyceae *Ankistrodesmus gracilis* (Reinsch) Korshikov, organismos do zooplâncton (*Dendrocephalus brasiliensis* (Pesta), *Artemia* sp, *Daphnia magna*, *Brachionus plicatilis* e copépodos) e uma espécie de peixe (bacalhau do Atlântico - *Gadus morhua*). Os organismos estudados são utilizados ou têm potencial para utilização em aquicultura.

A tese foi estruturada com uma introdução geral abordando a aquicultura, seguida de três capítulos em forma de quatro artigos científicos. No tópico *Conclusões,* buscamos integrar os resultados obtidos. Abaixo são apresentadas as visões gerais de cada capítulo.

No capítulo 1, apresenta-se o estudo da microalga Chlorophyceae de água doce, *Ankistrodesmus gracilis*, no qual se buscou avaliar a resposta bioquímica da alga às variações nas condições de cultivo. Esta microalga foi escolhida por ser utilizada como alimento para os organismos planctônicos, apresentando facilidade de cultivo e boas taxas de crescimento. O fósforo e o cobre foram os elementos escolhidos para variação nos tratamentos. O fósforo foi escolhido por ser um macronutriente essencial para a produção primária e por haver evidências experimentais de que certas espécies de algas, quando cultivadas em baixas concentrações de fósforo, podem acumular maiores quantidade de proteínas, lipídios ou carboidratos em suas células, o que representaria uma vantagem para utilização em sistemas aquícolas. O cobre foi escolhido por ser um micronutriente essencial às algas, porém exercendo toxicidade em concentrações acima das requeridas pelos organismos. Assim, além da análise da resposta bioquímica da clorofícea a diferentes concentrações desse elemento, também foram realizados testes para avaliação da toxicidade do mesmo para a alga. Além disso, dados da literatura indicam que as algas têm uma maior resistência aos tóxicos quando estão em um ambiente repleto de fósforo, ou seja, ambientes eutróficos podem mascarar os efeitos de contaminantes. Pensando nisso, foram também realizados testes de toxicidade com o metal cobre em função da concentração de fósforo no meio.

O capítulo 2 trata do estudo comparativo da composição bioquímica de três espécies de microcrustáceos com potencial para utilização como alimento vivo em cultivos de peixes e

crustáceos. O nosso principal interesse é na espécie nativa da região Nordeste do Brasil do Anostraca dulcícola *Dendrocephalus brasiliensis*, popularmente conhecida como branconeta. Este organismo tem grande potencial para uso como alimento vivo em aquicultura, particularmente em substituição à *Artemia* sp, cuja produção vem apresentando custos elevados nos últimos anos devido às crises na produção de cistos. O uso de *D. brasiliensis* poderia ser vantajoso especialmente em cultivo de peixes de água doce. Como a *Artemia* é um organismo de água salobra, ao ser introduzido em tanques com água doce, pode sofrer danos devido aos processos osmóticos. A despeito desse potencial de *D. brasiliensis*, poucas são as informações disponíveis sobre sua composição bioquímica e seus valores nutricionais. Assim, neste capítulo, abordamos a adequação bioquímica da branconeta para utilização como alimento vivo, comparativamente a outras duas espécies de microcrustáceos (*Daphnia magna* e *Artemia* sp.) normalmente utilizadas em aquicultura.

No capítulo 3 foram realizados experimentos controlados com *Gadus morhua* (bacalhau do Atlântico) para a determinação da influência de diferentes organismos alimentares (naturais ou enriquecidos) na composição lipídica e de aminoácidos dessa espécie. Os experimentos foram realizados usando protocolos estabelecidos para o cultivo da espécie, com o fornecimento de rotíferos (*Brachionus plicatilis*), *Artemia* sp. e zooplâncton natural como itens alimentares. Foram determinadas as composições lipídicas e de aminoácidos (livres ou totais) nos itens alimentares e no peixe.

RESUMO

A aquicultura é uma atividade de grande importância na produção de alimentos, porém apresenta como maior desafio o sucesso na sobrevivência dos peixes nas fases de pós-larva, geralmente relacionada à obtenção de alimento vivo, que possui uma composição bioquímica balanceada e essencial. *Artemia* sp. é utilizada com sucesso como alimento vivo, porém apresenta custo elevado e uma das alternativas para reduzir os custos é a utilização de espécies nativas, como o zooplâncton natural e o anostráceo dulcícola *Dendrocephalus brasiliensis*, filogeneticamente próximo à *A. salina.* O presente estudo avaliou como as alterações nas condições nutricionais afetam a alga *Ankistrodesmus gracilis*, a composição bioquímica de *D. brasiliensis, Artemia* sp. e *Daphnia magna* e como diferentes combinações de alimento afetam a composição bioquímica do bacalhau do Atlântico (*Gadus morhua*). Os resultados obtidos demonstraram que a disponibilidade de fósforo e cobre alteram a composição bioquímica das algas, além de afetar a produção de clorofila, taxas de crescimento e resistência ao metal; *D. brasiliensis* apresentou bons resultados em termos de conteúdo de carboidratos e proteínas, além de uma variação na composição lipídica de acordo com o sexo e estágio de desenvolvimento dos animais. Melhores resultados foram obtidos para o bacalhau quando alimentado com zooplâncton do ambiente natural acrescentado aos organismos cultivados e enriquecidos em tanques de cultivo. Com base nesses resultados, podemos concluir que o ambiente e disponibilidade de nutrientes, no caso das microalgas, afeta a composição bioquímica e capacidade de lidar com contaminantes. A composição bioquímica do zooplâncton pode afetar os níveis tróficos superiores, interferindo tanto na composição bioquímica quanto em desenvolvimento. Por tais motivos, é importante que se conheçam as condições ótimas de cultivo afim de se obterem os melhores resultados em cada nível trófico.

ABSTRACT

Aquaculture is an activity of great importance in food production, but fish survival in post- larval stages represents a big challenge. *Artemia* sp. is successfully used as live food, but is expensive and one of the alternatives to reduce costs is to use native species, such as natural zooplankton and fairy shrimp *Dendrocephalus brasiliensis,* phylogenetically close to *A. salina*. This study examined how changes in nutritional conditions affect the algae *Ankistrodesmus gracilis*, and the biochemical composition of *D. brasiliensis*, *Artemia* sp. and *Daphnia magna*. Moreover, we studied how different combinations of food affect the biochemical composition of Atlantic cod (*Gadus morhua*). The results showed that the availability of phosphorus and copper alter the biochemical composition of algae and affect the production of chlorophyll, growth rates and resistance to metal; *D. brasiliensis* showed good results in terms of the content of carbohydrates and protein, and a change in the lipid composition according to sex and stage of development of animals; cod showed better results when feed on wild zooplankton added to the cultivated and enriched organisms (rotifers and *Artemia*) in tanks. Based on these results, we conclude that the environment and nutrient availability in the case of microalgae, affects the biochemical composition and ability to handle contaminants. The biochemical composition of zooplankton can affect higher trophic levels, affecting both the biochemical composition. For these reasons, it is important to know the optimal cultivation conditions in order to achieve the best results in each trophic level.

1. INTRODUÇÃO

A aquicultura é o processo de produção, em sistemas de cultivos, de organismos aquáticos em qualquer estágio de desenvolvimento, desde ovos até adultos (Yflaar, 2003). Essa atividade produtora de alimentos tem se ampliado significativamente em todo o mundo, frente à necessidade de aumentar a produção protéica para melhorar a qualidade alimentar da crescente população mundial (Crispim *et al*., 1999). De acordo com Sipaúba-Tavares & Pereira (2008), a aquicultura brasileira ainda é carente de tecnologia para a produção de alimento vivo para ser fornecido às espécies de peixe cultivadas.

A maior dificuldade nessa atividade se encontra na obtenção de uma boa sobrevivência na fase inicial de larvas, onde ocorrem as maiores perdas (Luz & Zaniboni-Filho, 2001; Prieto et al, 2006). A qualidade e a quantidade de alimento adequado nos primeiros dias de vida dos peixes influenciam suas taxas de crescimento, tempo de maturidade sexual e longevidade (Qin et al, 1997). A melhor opção para a nutrição inicial de larvas de peixes é o alimento vivo devido ao conteúdo de ácidos graxos essenciais (Watanabe et al, 1984). A dieta inicial dos peixes também deve conter altos teores de aminoácidos livres, enzimas e água (Lazzaro, 1987), sendo que o zooplâncton preenche esses requisitos (Hebert, 1978).

Em ambientes naturais, os peixes conseguem balancear suas dietas escolhendo os alimentos que melhor suprem suas necessidades e dificilmente são observadas deficiências nutricionais nestes animais (Hamre, 2006). Os alimentos naturais possuem grande valor energético, com altos níveis de proteínas, minerais e vitaminas (Kubitza, 1997), melhorando o crescimento e sobrevivência de larvas de peixe (Furuya et al, 1999). Buscando obter melhores resultados de sobrevivência das larvas em ambientes artificiais, os organismos que servem de alimento podem ser enriquecidos com vitaminas e ácidos graxos essenciais para aumentar seu valor nutricional e proporcionar a transferência de elementos com melhor qualidade na cadeia trófica (Coutteau & Sorgeloos, 1997).

O zooplâncton, natural ou cultivado, possui ótimo valor nutricional como fonte de proteína e balanceamento de aminoácidos, minerais e lipídios, além de fornecerem enzimas que facilitam os processos de digestão e absorção dos nutrientes (Dabrowsky, 1984; Garcia-Ortega et al, 1998). Dentre os lipídios, os ésteres de cera são a maior reserva estocada em alguns crustáceos zooplanctônicos e uma importante fonte de energia para peixes (Olsen et al, 1991;

Houlihan et al, 2001). Sua composição bioquímica é alterada de acordo com o alimento ingerido (Rainuzzo et al, 1994; Brown et al, 1997; Sekino et al, 1997; Payne et al, 1998; Nanton and Castell, 1999, Pond & Tarling, 2011) e pode ser manipulada em sistemas de cultivo (Payne et al, 1998; Stottrup et al, 1999).

Os lipídios são moléculas ricas em carbono e com elevado valor energético, sendo importantes fontes de reserva para o zooplâncton marinho e de água doce. As microalgas são os produtores primários de lipídios e o zooplâncton atua como elo entre os produtores e os níveis tróficos superiores. A composição lipídica representa a integração entre a aquisição através do alimento e as perdas pela respiração e reprodução (Vanderploeg et al 1992), sendo influenciada por condições ambientais, como concentração de nutrientes (Breteler et al, 2005). Alguns constituintes dessas biomoléculas são nutrientes essenciais para os animais, tais como os ácidos graxos poliinsaturados (Parrish et al, 2005).

Os ácidos graxos apresentam importantes e diversificadas funções nos organismos, sendo requeridos em diferentes quantidades pelos vertebrados (Sargent et al, 1995). Por não serem degradados, os ácidos graxos podem ser usados como biomarcadores tróficos (Budge & Parrish, 1998; Arts et al, 2001; Iverson et al, 2004). Alguns ácidos graxos poliinsaturados (PUFA) são denominados ácidos graxos essenciais (EFA) por serem requeridos pelos organismos para desenvolvimento correto (Sargent et al, 1995) e saudável (Parrish, 2009). São considerados EFA os ácidos graxos 20:5ω3 (ácido eicosapentaenóico, EPA), 22:6ω3 (ácido docosahexaenóico, DHA), 20:4ω6 (ácido araquidônico, ARA) (Sargent et al, 1999a; b), e, de acordo com recentes estudos, 22:5ω6 (ácido docohexapentaenóico, ω6DPA) (Milke et al, 2006; Parrish et al, 2007; Garcia et al, 2008b).

Os EFAs são componentes das membranas fosfolipídicas, sendo importantes para a estrutura e o funcionamento celulares (Watanabe, 1993; Sargent et al, 1995), atividades metabólicas (Kainz et al, 2004), crescimento e sobrevivência (Watanabe, 1993; Sargent et al, 1995; Olsen et al 1997; Parrish, 2009), correto desenvolvimento visual e neural em larvas de peixes (Estevez et al, 1998; Sargent et al, 1999a; Arts et al, 2001) e também na imunidade e resistência, por serem precursores de eicosanoides, que atuam na resposta imune, em processos inflamatórios e de estresse (Sargent et al, 1995; Sargent et al, 1999a; Parrish et al, 2009). As razões em que os diferentes ácidos graxos são fornecidos também influenciam nas características

supracitadas, sendo o DHA, geralmente, requerido em maior quantidade em relação aos demais ácidos graxos (Sargent et al 1999b).

Os rotíferos, principalmente da espécie *Brachionus plicatilis,* são utilizados há um tempo como alimentação inicial das larvas de peixes marinhos, apresentando melhores resultados quando são enriquecidos (Evjemo & Olsen, 1997; Imsland et al, 2006; O'Brien-MacDonald et al, 2006; Park et al, 2006; Busch et al, 2011). Esta espécie, além de ter o cultivo bem estabelecido, apresenta características propícias para o uso como alimento vivo, como tamanho adequado para as larvas logo após a abertura da boca, reprodução e ciclo de vida rápidos, além de respostas rápidas aos enriquecimentos, refletindo em pouco tempo a composição bioquímica do alimento ingerido (Brown et al, 1997).

Os enriquecimentos de rotíferos e *Artemia* visam obter composições nutricionais semelhantes aos encontrados em copépodos naturalmente (Evjemo & Olsen, 1997) e esta composição desejada é importante para o sucesso das larvas (Watanabe, 1993; Bell et al, 2003). Geralmente os enriquecimentos são feitos com produtos comerciais, como DHA Selco™ (Evjemo et al, 2003), Super-Selco™ (Evjemo & Olsen, 1997; Garcia et al 2008a), Alga-Mac2000™ (Garcia et al 2008a; b; Olivotto et al, 2008), Ori-Green™ ou Ori-Culture™ (Busch et al, 2011) ou até mesmo com uso de óleos (Sargent et al 1999a; Evjemo et al, 2003) ou órgãos de peixes (Rainuzzo et al, 1994).

Os copépodos apresentam melhores composições nutricionais quando comparados à *Artemia* e rotíferos (Stottrup & Norsker, 1997; McEvoy et al, 1998), com altos níveis de astaxantina e vitaminas C e E (van der Meeren et al, 2008). Nos copépodos, a composição lipídica pode se alterar de acordo com a profundidade e pressão (Pond & Tarling, 2011) ou devido a variações sazonais (Clark et al, 2012). A composição de ácidos graxos do zooplâncton pode ser relacionada à de sua fonte alimentar (Stottrup & Jenssen, 1990; Sekino et al 1997; Payne et al, 1998; Clark et al, 2012). Devido à capacidade de incorporar, modificar e sintetizar os ácidos graxos, esses animais mantêm boas razões de DHA/EPA (Nanton and Castell, 1998; 1999; Drillet et al, 2011) e podem ser utilizados como marcadores tróficos (Parrish et al, 2000).

Os copépodos apresentam razões DHA/EPA próximo a 2, consideradas ideais para o desenvolvimento de larvas (Evjemo & Olsen, 1997; Sargent et al, 1999a; Stottrup et al, 1999; Bell et al, 2003). Tais razões só podem ser obtidas em rotíferos e *Artemia* enriquecidos (Rainuzzo et al, 1994; Evjemo & Olsen 1997; Evjemo et al, 1997). A pigmentação (Evjemo

&Olsen, 1997; Venizelos & Benneti, 1999), as taxas de sobrevivência e o crescimento de larvas de peixe podem ser afetadas pelo tamanho (Olsen et al 1999a) e qualidade do alimento ingerido (Payne et al, 1998; Kainz et al, 2004), sendo obtidos melhores resultados com o uso de copépodos, como única fonte alimentar ou como complemento ao uso de rotíferos e *Artemia* (Stottrup & Norsker, 1997; Payne et al, 1998; Stottrup, 2000; Olivotto et al, 2008). Embora os copépodos apresentem características superiores aos rotíferos e *Artemia* como alimento vivo, seu cultivo ainda não é bem estabelecido em grande escala (Stottrup, 2000; Drillet et al, 2011).

O crustáceo dulcícola *Dendrocephalus brasiliensis* Pesta 1921 (Anostraca: Thamnocephalidae), popularmente conhecido como branconeta, habita sistemas temporários de água doce e apresenta morfologia e sistema reprodutivo similares à *Artemia* sp. A sua distribuição ocorre naturalmente entre a Argentina e o Piauí (Calviño & Petracini, 2004), com relatos naturais nos estados Bahia, Piauí (Pesta, 1921); Rio Grande do Norte (Lutz, 1929); Ceará (Lopes, 2007), Minas Gerais (Passos, 2012) e também em Galápagos (Hartland-Rowe, 1966), sendo relatada como espécie exótica no estado de São Paulo (Mai et al, 2008).

A branconeta é uma espécie filtradora generalista, se alimentando de matéria em suspensão com bactérias e restos de matéria orgânica (Calviño & Petracini, 2004), parecendo preferir, no entanto, o fitoplâncton (Lopes, 2002). Seus predadores naturais são larvas e insetos adultos, além de peixes que habitam as lagoas temporárias (Coelho & Araújo, 1982). Embora tenha apresentado bons resultados ao ser fornecida como alimento a larvas de peixes e também ao camarão cinza (*Litopenaeus vannamei*) (Yflaar, 2003; Lopes, 2007), as pesquisas sobre os processos biológicos e ecológicos desse animal encontram-se em fase inicial, sendo que pouco é conhecido também sobre o seu valor nutricional. As suas características, tais como bom tamanho ao eclodir, podendo ser facilmente capturada pelas larvas de peixe, e fácil cultivo em tanques (Lopes, 2002) fazem dessa espécie um organismo de interesse como alternativa ao uso como alimento vivo na aquicultura, especialmente no Brasil e em outros países tropicais, devido às altas temperaturas (26 – 30ºC) necessárias para o seu desenvolvimento.

As algas, utilizadas como alimento pela comunidade zooplanctônica e por algumas espécies de peixe, podem sofrer alterações na sua composição bioquímica quando se encontra limitada por nutrientes, especialmente fósforo e nitrogênio, resultando em maiores valores de lipídios e carboidratos, e menores de proteína, quando comparadas às algas em condições ótimas de crescimento (Alcoverro et al, 2000; Bhola et al, 2011; Chia et al, 2013a, Geider & LaRoche,

2002). Como consequência dessas alterações, a biota que se alimentar dessas algas com diferentes valores nutricionais será afetada, sendo observadas alterações na taxa de filtração e ingestão de algas que estão limitadas por algum nutriente (Kilham et al, 1997). A microalga *Ankistrodesmus gracilis* foi escolhida para esse estudo por ser de fácil cultivo e crescimento rápido, além de ser utilizada com sucesso como alimento para o zooplâncton na aquicultura.

Com o intuito de avaliar como as mudanças nas condições ambientais podem afetar a resposta da biota aquática, esta pesquisa enfocou as alterações bioquímicas em diferentes níveis tróficos aquáticos. A microalga *Ankistrodesmus gracilis* foi cultivada com diferentes disponibilidades de fósforo e cobre, elementos essenciais para o correto metabolismo da alga, mas cujo fornecimento impróprio pode afetar o desenvolvimento da comunidade. Objetivando a obtenção de novas alternativas para o fornecimento de alimento vivo na aquicultura e um maior conhecimento das propriedades nutricionais do zooplâncton, diferentes espécies de microcrustáceos foram avaliadas quanto à composição bioquímica, buscando as melhores características para cultivo e alimentação de peixes. Em outro elo da cadeia, avaliou-se como a composição bioquímica do alimento fornecido aos peixes influenciam seu desenvolvimento e sua composição bioquímica.

2. OBJETIVOS

O objetivo principal deste estudo foi avaliar a composição bioquímica de organismos aquáticos de diferentes níveis tróficos, sob diferentes condições ambientais, visando à utilização em aquicultura. Para tanto, foram realizados os seguintes estudos específicos:

1) Avaliação do impacto da manipulação nutricional na composição bioquímica de microalgas: concentração de proteínas, carboidratos e lipídios em células da clorofícea *Ankistrodesmus gracilis* em função de diferentes concentrações de fósforo e de cobre no meio.
2) Determinação do conteúdo de carboidratos, lipídios e proteínas em*D. brasiliensis* comparativamente a outras espécies de microcrustáceos comumente utilizadas como alimento vivo em aquicultura (*Artemia* sp. e *Daphnia magna*).
3) Avaliação qualitativa dos lipídios que compõem o corpo das espécies estudadas, através da determinação das seguintes classes lipídicas: hidrocarbonetos alifáticos (nonadecano), ceras (estearil palmitato), triglicerídeos (tripalmitina), ácidos graxos livres (ácido palmítico), esterol (colesterol), lipídios polares móveis em acetona (monopalmitina) e fosfolipídios (lecitina).
4) Avaliação da influência de diferentes tipos de alimento (naturais e enriquecidos) na composição lipídica e de aminoácidos do bacalhau do Atlântico (*Gadus morhua*).

3. METODOLOGIA

Laboratório

Cultivo de algas

A alga *Ankistrodesmus gracilis* (Fig 1) foi obtida junto ao Departamento de Botânica, da Universidade Federal de São Carlos e cultivada em meio LC Oligo (Tabela1) até obtenção de cultura estoque suficiente para a realização dos experimentos. Após o preparo das soluções estoque do meio de cultura, as mesmas foram mantidas sob refrigeração e para o preparo do meio de cultura, eram adicionados 1 mL das soluções 1, 2, 3, 4 e 7 e 0,5 mL das soluções 5 e 6. O meio então tinha o pH ajustado para 7 e autoclavado a 121 °C por 20 minutos. Após o tempo mínimo de 24 horas na temperatura ambiente, o meio era exposto à radiação UV em cabine de fluxo laminar e então as algas eram inoculadas. O cultivo foi mantido em sala com temperatura (22 ± 2 °C); iluminação (1000 lux) e fotoperíodo (16h:8h luz/escuro) constantes.

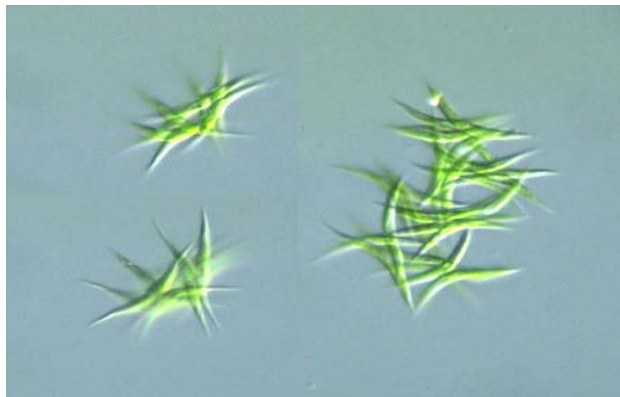

Figura 1: Microalga clorofícea *Ankistrodesmus gracilis*

Fonte: http://protist.i.hosei.ac.jp/pdb/images/Chlorophyta/Ankistrodesmus/sp_5b.jpg

Tabela 1. Meio de cultura Oligo (AFNOR, 1980). ABNT, 2005.

Solução	Reagente	Quantidade (mg)	Preparo
1	$Ca(NO_3).4H_2O$	4 000	Em 100 mL de água destilada
2	KNO_3	10 000	Em 100 mL de água destilada
3	$MgSO_4.5H_2O$	3 000	Em 100 mL de água destilada
4	K_2HPO_4	4 000	Em 100 mL de água destilada
5	$CuSO_4.5H_20$	30	Em 1000 mL de água destilada
	$(NH_4)6Mo7O_{24}.H_2O$	60	
	$ZnSO_4.7H_2O$	60	
	$CoCl_2.6H_2O$	60	
	$Mn(NO_3)_2.4H_2O$	60	
	$C_6H_80_7.H_20$	60	
	H_3BO_3	60	
6	$C_6H_5FeO_7.5H_2O$	1 625	Em 1000 mL de água destilada
	$FeCl_3.6H_20$	6 25	
	$FeSO_4.7H_2O$	6 25	
7	$NaHCO_3$	15 000	Em 1000 mL de água destilada

A cada 24 h foram retiradas alíquotas de cada tratamento para a o acompanhamento do crescimento algal e as amostras para contagem de células foram fixadas com lugol acético. A determinação da densidade celular foi realizada através de contagem de células ao microscópio óptico (Leica, DMLS) utilizando-se um hemocitômetro Improved Neubauer-Bright Line.

A clorofila *a* foi determinada de acordo com a metodologia descrita em Shoaf & Lium (1976) em amostras de 10 mL filtradas em filtros de éster de celulose (045 µm poro, 25 mm diâmetro). Após extração do pigmento, a densidade óptica da amostra foi determinada em espectrofotômetro (HACH DR 5000) nos comprimentos de onda 664 e 647 nm, tendo sido utilizado como branco um filtro sem algas submetido ao mesmo processo de extração do pigmento. Os valores de clorofila *a* (µg.mL-1) foram calculados de acordo com a equação (Jeffrey & Humphrey, 1975):

$$Chl\ a = (11.93 \text{ x } \lambda 664 - 1,93 \text{ x } \lambda 647) \text{ x } \frac{\text{volume solvente}}{\text{volume filtrado}}$$

onde: λ664 = absorbância para o comprimento de onda de 664 nm; λ647 = absorbância para o comprimento de onda de 647 nm.

Cultivo de **Dendrocephalus brasiliensis**

Foram realizadas tentativas de eclosão e cultivo de *D. brasiliensis* em béqueres e aquários no laboratório e em caixas d'água na Estação de Piscicultura do Departamento de Hidrobiologia (DHb) da Universidade Federal de São Carlos. Em testes preliminares foram coletados indivíduos já eclodidos em tanques experimentais e aclimatados gradativamente à água reconstituída (ABNT, 2005) e os cistos depositados por esses animais foram secos e eclodidos em água reconstituída e água filtrada. Em condições controladas de luz (fotoperíodo 12/12h claro/escuro) e temperatura (25 ± 1 °C), foram fornecidas algas previamente centrifugadas, na concentração de 2 x 10^5 células/mL.

Análise da composição bioquímica dos organismos

As análises bioquímicas foram realizadas para verificar possíveis mudanças na composição bioquímica dos organismos de acordo com as condições de cultivo.

Análise de Carboidratos Totais

A determinação de carboidratos totais no material particulado, tanto do zooplâncton como do fitoplâncton seguiu o método descrito em Liu et al (1973), que é a modificação da técnica descrita em Dubois et al (1956). Tal metodologia tem como base a reação com fenol e ácido sulfúrico no material particulado. Em tubos de centrífuga foram acrescentados a amostra a ser analisada, 1 mL de água destilada e 1 mL de solução de fenol e misturado em vórtex. Adicionou-se 5 mL de H_2SO_4 concentrado e as amostras foram deixadas em uma bandeja com gelo durante 10 minutos e, decorrido este tempo, foram centrifugados a 4400 rpm por 10 minutos. Após a centrifugação, o sobrenadante foi transferido para cubetas com o uso de pipeta de Pasteur e lido no espectrofotômetro a 485 nm. Foram feitas curvas de calibração a partir de concentrações conhecidas e o branco é composto de água, fenol e ácido sulfúrico.

Análise de Proteínas Totais

A determinação do conteúdo total de proteína no material particulado, tanto do zooplâncton como do fitoplâncton seguiu uma adaptação das metodologias descritas em Bradford (1976) e Rausch (1981). Foi preparado um reagente para determinar as proteínas (Reagente de Proteína) dissolvendo 100 mg de Coomassie Brilliant Blue® G-250 (Sigma) em 50 mL de etanol 95%. A esta solução é adicionado 100 mL de ácido fosfórico 85%. A solução resultante será diluída até o volume final de 1 litro. A solução de proteína é feita com o uso da albumina bovina duas vezes cristalizada (Bovine serum albumin 2 x crystallized) em NaOH 0,5 N.

Para a extração das proteínas, foram centrifugados 5 mL de amostra a 1500 rpm durante 5 minutos. O pellet formado foi ressuspendido em 1,5 mL de 0,5N NaOH e a amostra aquecida por 1 hora a 80°C. A amostra foi resfriada em água corrente até a temperatura ambiente, então centrifugada por 15 minutos a 4400 rpm e com o sobrenadante transferido para tubos graduados. A 50 µL do sobrenadante foram adicionados 2,5 mL do reagente de proteína. Após a estabilização de cor (geralmente de 5 a 20 minutos), foi efetuada a leitura no comprimento de onda 595 nm. Os padrões são feitos a cada dia de análise a partir de concentrações conhecidas de proteínas com o uso do padrão de albumina.

Análises qualitativa das classes de lipídeos

A detecção de diversas classes lipídicas foi feita através de cromatografia de camada delgada com detecção por ionização em chama (TLC/FID), com o uso do Iatroscan™ Mark VI. As classes lipídicas detectadas com esta metodologia são: hidrocarbonetos alifáticos (HC), éster de cera (WE/SE), cetona (Ket), triglicerídeos (TAG), ácidos graxos livres (FFA), álcool alifático (ALC), esterol (ST), lipídios polares móveis em acetona (AMPL) e fosfolipídios (PL).

Foram analisados os lipídios particulados a partir das células em cultura e diretamente dos organismos do zooplâncton. As amostras de alga foram filtradas em filtros de fibra de vidro previamente calcinados (400°C, 24 horas) e a extração foi feita com uso de clorofórmio e metanol (Parrish, 1987; 1999), além do uso de um sonicador para romper as células. Brancos de procedimento foram realizados em cada dia de amostragem.

Os detalhes de extração e análise dos lipídios estão presentes nos capítulos de composição bioquímica, uma vez que houve pequenas variações quanto à metodologia de extração, especialmente a quantidade de solvente necessário para a realização do procedimento.

Peso seco

Filtros de fibra de vidro foram calcinados a 400°C por 8 horas em forno mufla, colocados por 1 hora no dessecador e, pesados em balança microanalítica de alta precisão (Sartorius MC21S, com acuracidade de 1 μg). Para a obtenção dos valores de peso seco foram filtrados 75 mL da cultura algal em fase exponencial. Os filtros foram deixados em estufa a 60°C por 24 horas, ou até obter peso constante, sendo mantidos no dessecador por 1 hora antes de efetuar a pesagem.

Estação de aquicultura do Departamento de Hidrobiologia

Na estação de aquicultura do Departamento de Hidrobiologia da Universidade Federal de São Carlos foram mantidos cultivos estoque de *Daphnia magna* (Fig 2), *Dendrocephalus brasiliensis* (Fig 3) e *Artemia salina* (Fig 4), que se mantiveram alimentados a partir de algas presentes nos próprios tanques de cultivo dos animais.

Figura 2: *Daphnia magna*

Fonte: http://www.pondscape.be/picpondlife.html

Figura 3: *Dendrocephalus brasiliensis*

Figura 4: *Artemia salina*

http://www.hlasek.com/foto/artemia_salina_bh0173.jpg

4. CAPÍTULOS

CAPÍTULO 1

Os efeitos do cobre e fósforo na composição bioquímica e toxicidade deste metal para a microalga Ankistrodesmus gracilis

O cobre (Cu) é um micronutriente essencial ao fitoplâncton mas pode ser tóxico quando presente em concentrações mais elevadas do que o requerido para os organismos (Wang e Dei , 2001). Sua principal ação nas microalgas está relacionada à ativação enzimática, na qualidade de co-fator. É também importante nos processos fotossintéticos, onde tanto a falta como o excesso são prejudiciais (Lombardi e Maldonado, 2011).

No ambiente, o cobre apresenta elevada afinidade por ligantes orgânicos dissolvidos (Tonietto et al, 2014), e por grupos funcionais em biomoléculas nos organismos (Mason e Jenkins, 1995). Essa alta afinidade faz com que o cobre tenha sua especiação grandemente afetada pelo tipo de ambiente e organismos ali presentes, o que pode influenciar diretamente sobre a disponibilidade e toxicidade ao fitoplâncton (Lombardi et al, 2002).

O fósforo (P) é um dos nutrientes de grande importância ao fitoplâncton e, em ambientes aquáticos, pode controlar a dinâmica desses organismos, por isso é muitas vezes o nutriente chave em situações de controle da eutrofização. Seu excesso induz a proliferação de microalgas, levando a consequências prejudicais ao ecossistema. Para o fitoplâncton, o P é requerido para o

metabolismo energético e constituinte dos ácidos nucleicos (Beardall et al., 2005; Omelon and Grynpass, 2008; Rhee, 1973). Sua limitação afeta o processo fotossintético, divisão celular e a composição bioquímica das células algais (Alcoverro et al., 2000; Berdalet et al., 1994; Bertilson et al., 2003; Cembella et al., 1984; Granum et al., 2002; Guschina and Harwood, 2006; Khozin-Goldberg and Cohen, 2006; Lai et al., 2011; Zhao et al., 2009; Chia et al., 2013a; 2013b).

Considerando que o fitoplâncton apresenta plasticidade fisiológica, e.g., adapta-se à ampla variedade de condições ambientes através da síntese de diferentes biomoléculas, como os lipídios, proteínas e carboidratos, a interação entre o P e o Cu pode levar a alterações da composição bioquímica, com aumento da síntese de carboidratos e lipídios, como mostrado em Chia et al. (2013a) para o metal Cd e o P. Em situações controladas, a interação entre os dois elementos (Cu e P) pode ser usada para a manipulação da composição bioquímica celular, mas no ambiente natural, onde essa interação depende de vários fatores, uma alteração da energia que é transferida na cadeia alimentar pode ter consequências negativas aos organismos de níveis tróficos superiores, como o zooplâncton e peixes.

Uma vez que diferentes biomoléculas são sintetizadas pelo fitoplâncton sob diferentes condições, a variação na concentração de P e de Cu, assim como a interação entre eles, pode induzir à síntese de compostos de interesse na aquicultura, como por exemplo aumento de ácidos graxos insaturados ou outros lipídios nas células algais.

Neste primeiro capítulo, buscou-se detectar e compreender os efeitos do cobre, do fósforo e de várias combinações fósforo/cobre na fisiologia e toxicidade do metal sobre a microalga. Assim, dividiu-se este capítulo em duas partes, uma primeira que investigou a composição bioquímica de *A. gracilis* e uma segunda parte onde se apresenta resultados sobre os efeitos da interação cobre-fósforo na toxicidade para a microalga. Os dois estudos apresentados neste capítulo são listados abaixo.

1.1 The effects of phosphorus and copper in the biochemical composition of Ankistrodesmus gracilis (Reinsch) Korshikov

1.2 *The importance of phosphorus on copper toxicity to Ankistrodesmus gracilis (Reinsch) Korshikov*

1.1

The effects of phosphorus and copper in the biochemical composition of *Ankistrodesmus gracilis* (Reinsch) Korshikov

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Abstract

Microalgae require several nutrients for its healthy growth. If these requirements are not reached, phytoplankton community can be altered, affecting aquatic food chains. In the present study, two essential nutrients were altered simultaneously, phosphorus (P) and the trace metal copper (Cu). Phosphorus effects on the biochemical composition (proteins, carbohydrates and lipid classes) were evaluated considering a P range of 2.3×10^{-4} (control) to 2.3×10^{-6}mol. L^{-1} and Cu ions from 6.9×10^{-9} mol L^{-1} to 6.1×10^{-8}mol. L^{-1}. The Chlorophyceae *Ankistrodesmus gracilis* was used as test organism. Algal cells were acclimated to each P concentration prior to Cu exposure that occurred for 120 hours. Moderate P limitation and high Cu increased proteins and carbohydrates content in the microalgae and phosphorus depleted cells were more sensitive to Cu ions than phosphorus replenished cells. Lipids synthesis was highly affected by phosphorus concentration, with the highest lipid production in the lowest P concentration. The most affected lipids were triacylglycerol and phospholipids, especially when comparing phosphorus limited cells with controls.

Key words: copper; orthophosphate; lipid; Chlorophyceae; algal culture; algae biochemistry.

1 - Introduction

Algae need sufficient amount of nutrients for their healthy metabolism. Among these nutrients, phosphorus is of great importance, because of its role in the energetic metabolism and in nucleic acids (Beardall et al., 2005). Phosphorus limitation affects cell division, chlorophyll *a* production and photosynthesis, as demonstrated in Alcoverro et al. (2000) and Cembella et al. (1984), as well as the synthesis of molecules such as proteins, carbohydrates and lipids (Lombardi and Wangersky, 1992; Guschina and Harwood, 2006; Khozin-Goldberg and Cohen, 2006; Zhao et al., 2009; Lai et al., 2011).

Proteins can constitute up to 60% of cell mass under healthy conditions (Geider and La Roche, 2002), but in nutrient limited conditions, carbohydrates and lipids are usually higher than proteins (Bertilson et al., 2003; Kilham et al., 1997; Shifrin and Chisholm, 1981). The proteins: carbohydrates ratio can be used to evaluate the nutritional status of the cells and a decrease in

this ratio indicate that culture may be under nutrient limitation (Ganf et al., 1986; Kilham et al., 1997; Lai et al., 2011; Lizotte and Sullivan, 1992).

Copper is a nutrient required for the metabolic processes, such as cell division, respiration and photosynthesis (Lombardi and Maldonado 2011; Bossuyt and Janssen, 2004, 2005; Droppa et al., 1984), but in concentrations higher than required, these processes are negatively affected (Baumann et al., 2009; Hall et al., 1989; Lombardi and Maldonado, 2011; Rodgher et al., 2008; Sunda and Huntsmann, 1998; Tripathi and Gaur, 2006; Wang and Dei, 2001). Metal exposure can lead to changes in biochemical composition, reducing protein content and altering lipid metabolism (Kumar et al., 2010; Pinto et al., 2011), structural organization (Einicker-Lamas et al., 2002) and modifying ion balance (Jensen et al., 1982) in algae. According to the results of Sivakumar et al. (2010), the metal competes with nutrients for binding sites inside the cell and can exert toxic effects.

It has been shown that the biochemical composition of microalgae can reflect nutrient conditions they are exposed to (Beardall et al., 2001; Lai et al., 2011) or even the senescence of cultures (Pratt and Johnson, 1963). The influence of phosphorus availability in copper sensitivity in algae has also been shown (Guasch et al., 2004; Serra et al., 2010), as well as the biochemical composition under different phosphorus (Kilham et al., 1997; Lombardi and Wangersky, 1991) or copper (Einicker-Lamas et al., 2002; Guschina and Harwood, 2006) concentration, but studies in the biochemical responses of microalgae to copper and phosphorus concentrations varying are scarce. The aim of this study was to evaluate the influence of these two essential nutrients on the biochemical composition considering the synthesis of total lipids, proteins and carbohydrates in the freshwater microalga *Ankistrodesmus gracilis*.

2 - Materials and methods

2.1 – Algae cultures

The freshwater microalgae *Ankistrodesmus gracilis* (Chlorophyceae; 005 CH) was obtained from the culture collection of the Botany Department at Federal University of São Carlos, (SP, Brazil). The culture was maintained in L.C. Oligo medium (AFNOR, 1980) at pH 7.0 and autoclaved for 20 minutes at 121°C. Erlenmeyers with 2000 mL of capacity with 1000

mL of medium was used to maintain the cells. The cultures were kept in controlled conditions of light intensity (130 mmol m^{-2} s^{-1}), photoperiod (16:8 light: dark) and temperature (22°C ± 2).

Experimental cultures were performed with algae previously acclimated to 5 different concentrations of phosphorus for at least 45 days, with 10 partial renewals of medium, in semi continuous cultures (2.3 x 10^{-4} – control; 1.1 x 10^{-4}; 2.3 x 10^{-5}; 4.6 x 10^{-6}; 2.3 x 10^{-6}mol. L^{-1}). The acclimation is necessary to the algae reflect the actual nutrient available in the medium. After acclimation, algal cells were inoculated into medium containing different copper concentrations (0.7 – control; 1.2; 3.0 and 6.1 x 10^{-8}mol. L^{-1} Cu^{2+}), to which they were exposed for 120 hours. After this period, samples were collected for biochemical analysis. Cultures were performed with three experimental replicates. The control concentrations of phosphorus and copper are the recommended in the LC Oligo medium.

2.2 – Biochemical analysis

All glassware were first washed with tap water and neutral detergent, rinsed with tap water and placed in10% hydrochloric acid for 7 days, after which they were rinsed with deionized and ultra-pure water. Whenever glass fiber filters (GF/C; Boeco, Germany) were used, they were previously burned at 400°C for24h. The glassware used for lipid analysis were burned at 400°C for12h and rinsed with methanol and chloroform just before use.

Proteins

Proteins were quantified according to Bradford (1976) using a calibration curve performed with bovine serum albuminum as standard. Culture samples (10 mL) were centrifuged at 1500 rpm for 10 minutes (Eppendorf 5702R, Germany), the supernatant was removed and the pellet resuspended in 1.5 mL of NaOH 0.5 N. These were further incubated in an oven at 100 ^{o}C for 1 hour for protein extraction. After this, the samples were centrifuged at 4400 rpm for 15 min and the supernatant used for protein determination. Protein reagent (0.01% Coomassie Blue, 4.7% ethanol and 8.5% phosphoric acid) was added and the absorbance at 595 nm measured (HACH DR 5000; HACH Company, USA).

Carbohydrates

Total intracellular carbohydrates were determined using the modified phenol-sulphuric method according to Liu et al. (1973). Samples (10 mL) were centrifuged at 1500 rpm for 10 minutes (Eppendorf 5702R, Germany), the supernatant was discarded and the pellet used for the determination of carbohydrates. For measurement of the reaction color, the samples were centrifuged at 4400 rpm for 10 minutes and the supernatant read at 485 nm against blank reagent. Carbohydrates quantitation was based in calibration curves using glucose as standard.

Lipids

Total lipids and lipid classes were performed through thin layer chromatography with flame ionization detection(TLC/FID)using anIatroscanMK6 (Mitsubishi Kagaku Iatron Inc., Tokyo, Japan) according to the methodology described in Parrish (1999) that is based on Folch et al (1957).Culture samples were filtered through glass fiber filters that were grinded with a glass rod and lipid extraction was done using chloroform:methanol:chloroform extracted water (2:1:1), sonicated for 5 minutes (Unique Group, Indaiatuba, Brazil) and centrifuged for 2 minutes at 3000 rpm (Eppendorf 5702R, Germany). The organic layer containing lipids was removed and transferred to a vial, then 6 mL chloroform were added and the procedure repeated 3 times. Samples were concentrated under ultrapure N_2, sealed and stored at -20°C until analysis. For the chromatography (TLC/FID), samples (and standards) were spotted onto quartz rods (Chromarod SIII) using a Hamilton syringe. The samples were focused twice in 100% acetone and placed in a constant humidity chamber for 5 minutes. Three solvent systems were used for the complete sample development that resulted in the detection of 9 lipid classes. The first solvent system was composed of hexane:diethyl ether:formic acid (98.95:1:0.05), the second was hexane:diethyl ether:formic acid (79:20:1) and the third was chloroform:methanol:chloroform extracted water (5:4:1). After each development, the rods were kept in the Iatroscan for 5 minutes before scanning, and for 5 minutes in the humidity chamber after scanning. Lipid classes were identified from calibration curves made with lipid standards obtained from Sigma-Aldrich (USA). The analytical conditions for the TLC-FID runs were: hydrogen flow 173 mL min^{-1}, air flow 2 L min^{-1} and scan speed 4 mm s^{-1}.

Data analysis

Proteins, carbohydrates and lipids data were submitted to normality and homogeneity analysis, ANOVA test and Tukey's post hoc ($p < 0.05$), using GraphPad Instat 3.0 software.

3 - Results and Discussion

Microalgae are the base of aquatic food webs and any variation in its protein content can consequently affect organisms of higher trophic levels that feed on them. The results observed in control and 1% P are similar to those presented in Berdalet et al (1994), where phosphorus starved cells did not show difference in protein/cell, but in moderate phosphorus limitations (50, 10 and 2% P), there was an increase in protein concentration. This is also in accordance with the results presented in Chia et al (2013a) that analyzed different phosphorus concentrations in *Chlorella vulgaris.* But, these results are different from those obtained by Zhao et al (2009), who showed reduction in protein content in *S. costatum* grown under phosphorus limitation.

Treatments with 50% P in relation to the control presented the highest protein content, as shown in Table 1. For this P concentration, no effect of copper ions in the synthesis of proteins was detected (ANOVA, $p > 0.05$). However, no significant correlation between protein content and P concentrations was observed. In the present research there was an increase in protein in the most toxic scenario of copper in phosphorus control treatment. On the other hand highest values of protein were observed under combined effects of phosphorus limitation and copper exposure, especially in moderate conditions (50 and 10% P and 3 x 10^{-8} M Cu^{2+}). Compared to control of copper, the trend was the increase of protein with more copper in the medium in the phosphorus-limited treatments.

Similarly to our results, Chia (2011) exposed *Chlorella vulgaris* to different phosphorus and cadmium treatments and observed highest values of proteins in moderate limitation of phosphorus and cadmium. Sathya and Balakrishnan (1988) also observed highest protein values in algae exposed to cadmium when compared to controls.

The results for total carbohydrates content in *A. gracilis* are shown in Table 2. Although no significant correlation between P and carbohydrates concentration was obtained, the highest carbohydrate content was obtained in the cultures with the lowest P concentration. Similarly, no correlation was observed between copper concentration and carbohydrates in *A. gracilis*.

Table 1. Protein concentration (pg.cell^{-1}) I n*Ankistrodesmus gracilis* for different combinations of free copper ions and total phosphorus. Values represent mean of n=3 ± SD. Rows with the same alphabets are not significantly different ($p < 0.05$).

Cu^{2+} (x 10^{-8} mol.L^{-1}) / P (mol.L^{-1})	0.69	1.2	3	6.1
2.3×10^{-4} (C)	3.1 ± 0.2[a]	3.1 ± 0.4[a]	3.2 ± 0.4[a]	5.3 ± 0.2[b]
1.1×10^{-4} (50% P)	6.6 ± 0.3[c]	7.2 ± 0.5[c]	8.2 ± 0.4[c]	7.1 ± 0.2[c]
2.3×10^{-5} (10% P)	5.5 ± 0.4[b]	5.6 ± 0.3[b]	7.8 ± 0.7[c]	5.3 ± 0.8[b]
4.6×10^{-6} (2% P)	4.4 ± 0.1[b]	3.1 ± 0.4[a]	4.4 ± 0.3[b]	5 ± 0.3[b]
2.3×10^{-6} (1% P)	3.3 ± 0.4[a]	3.4 ± 0.1[a]	4.8 ± 0.3[b]	3.2 ± 0.1[a]

It is known that nutrient deficiency can increase the production of carbohydrates (Chia et al., 2013; Alcoverro et al, 2000). Our results are in accordance to literature. We obtained carbohydrate increase in phosphorus-limited conditions with regular copper concentrations present in LC Oligo medium. Similarly to the results of Chia et al. (2013a), we obtained the highest carbohydrate concentration in cells acclimated to the more extreme phosphorus limitation (1% P treatment).

The effects of copper in carbohydrates production were more significant in samples acclimated to copper concentrations higher than the control. Significant variations were obtained in the treatments where phosphorus was lower than the control and copper higher. These results suggest that phosphorus limitation has more impact in carbohydrate production than copper exposure.

Table 2: Carbohydrate concentration (pg.cell^{-1}) in *Ankistrodesmus gracilis* for different combinations of free copper ions and total phosphorus. Values represent mean of n=3 ± SD. Rows with the same alphabets are not significantly different ($p < 0.05$).

Cu^{2+} (x 10^{-8} mol.L^{-1}) / P (mol.L^{-1})	0.69	1.2	3	6.1
2.3×10^{-4} (C)	4.3 ± 0.5[a]	5.3 ± 0.2[a]	4.8 ± 0.3[a]	4.9 ± 0.2[a]
1.1×10^{-4} (50% P)	5.8 ± 0.3[a]	6.3 ± 0.4[a,c]	11.7 ± 1.1[b]	4.2 ± 0.3[a]
2.3×10^{-5} (10% P)	4.7 ± 0.4[a]	5 ± 0.7[a]	10.9 ± 0.1[b]	8.8 ± 0.6[d]
4.6×10^{-6} (2% P)	6.7 ± 0.5[a,c]	4.7 ± 0.4[a]	4.7 ± 0.1[a]	4.8 ± 0.2[a]
2.3×10^{-6} (1% P)	11.9 ± 0.2[b]	14.7 ± 0.9[e]	7.6 ± 0.6[d]	7.7 ± 1.3[c,d]

Lipid classes for *A. gracilis* are shown in Figure 1. Major lipids were triacylglycerol (TAG) and phospholipids (PL) independent of phosphorus and copper conditions. Hydrocarbons were present in less than 5% of the total lipids, however under in $2.3x10^{-5}$ mol L^{-1} acclimated cells exposed to Cu concentrations of 3×10^{-8} and 6×10^{-8} mol L^{-1}, HC was present in approximately 20% of the total lipids.

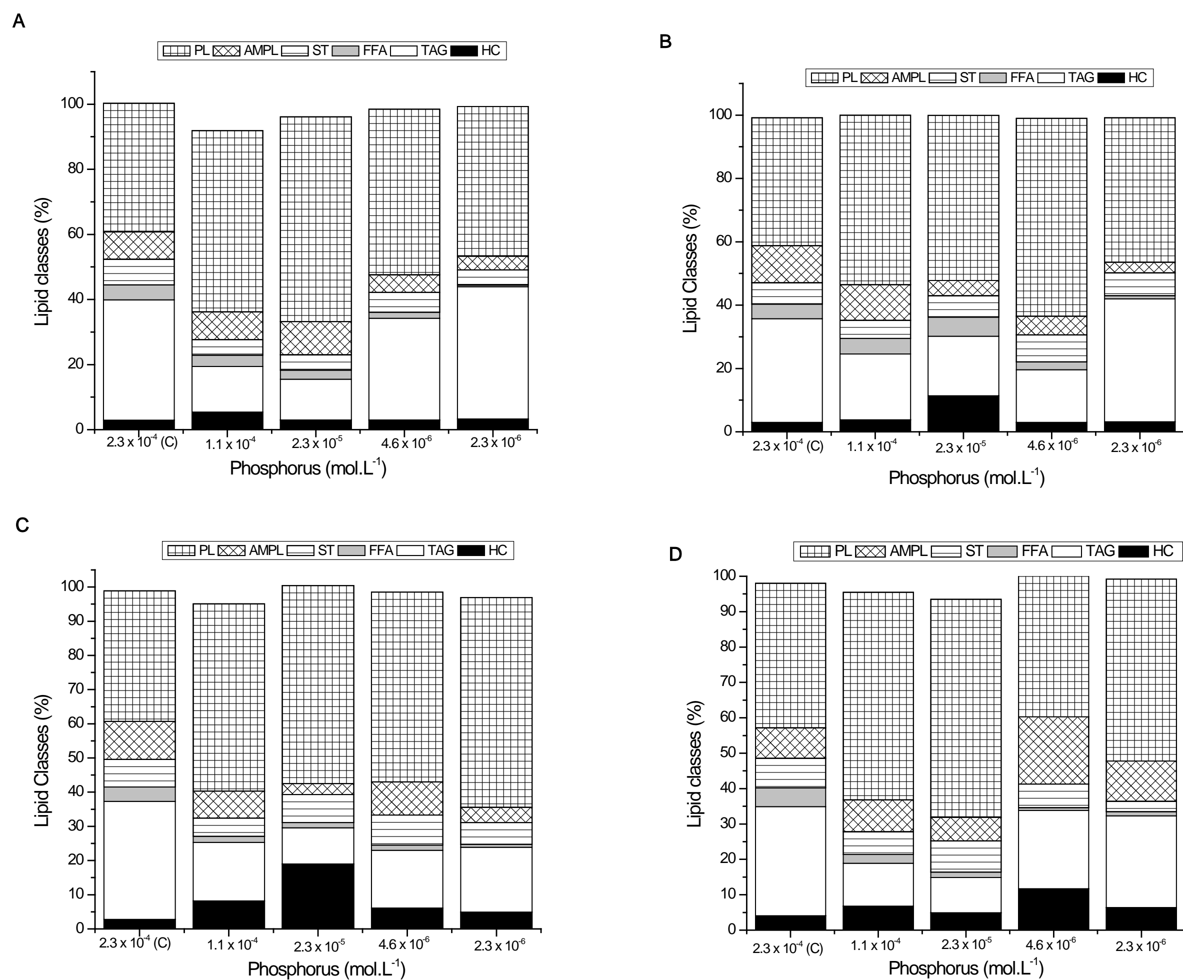

Figure 1. Relative lipid class composition (%) for *Ankistrodesmus gracilis* acclimated to different phosphorus concentrations and exposed to different copper treatments. (A) 0.69 x 10^{-8} M Cu^{2+} (control); (B) 1.2 x 10^{-8} M Cu^{2+}; (C) 3 x 10^{-8} M Cu^{2+} and (D) 6 x 10^{-8} M Cu^{2+}.

Lipid class composition was mostly affected by 50 and 10% phosphorus treatments, but in extreme P limitation (2 and 1% P), different behaviour was obtained. In phosphorus control treatment, significant changes were observed only in 10^{-7} M Cu^{2+} (data not shown), increasing the contributions of HC and PL, and reducing percentages TAG, FFA, ST and AMPL. In 50% P treatment, significant differences were not observed according to copper concentration, but compared to phosphorus control, there was a reduction in TAG (37% to 14%) and an increase in PL (39 to 56%) classes. In the other phosphorus treatments, the presence of copper affected the contribution of lipid classes, especially increasing HC and AMPL and reducing TAG in treatments with more copper.

The results of the present study agree with those observed by Chia et al (2013b) for another chlorophyceae. In phosphorus limitation, the authors obtained increase in HC and decrease in ST and AMPL classes in *Chlorella vulgaris*, but an increase in TAG and decrease in PL were obtained. In *A. gracilis,* no increase in TAG was obtained. Chia et al (2013b) also observed that lipid class's alterations were more related to phosphorus concentrations than cadmium.

According to the results, the most significant changes in biochemical composition of *A.* gracilis were related to phosphorus, not copper. In most extreme scenarios tested, i.e., high copper and low phosphorus, occurred the more notable changes, especially related to carbohydrates and lipids.

4 - Conclusion

The combinations of moderate copper stress (3 x 10^{-8} M Cu^{2+}) and 50 % and 10 % phosphorus resulted in the highest values of carbohydrates, proteins and lipids. Based on the results, in general, we can conclude that phosphorus limitation had a higher impact in biochemical composition than copper stress.

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1.2

The importance of phosphorus availability during algal growth on copper toxicity to *Ankistrodesmus gracilis* (Reinsch) Korshikov

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Abstract

Microalgae need a variety of nutrients at specific concentrations to allow optimal growth. If these concentrations are modified, the development of the phytoplankton community and the aquatic food chain can be affected. In the present study two essential nutrients were altered, phosphorus (P) and the trace metal copper (Cu). We assessed the effects of P (2.3×10^{-4} – control, 1.1×10^{-4}, 4.6×10^{-6} and 2.3×10^{-6}mol. L^{-1}) and free copper ions concentrations (6.9×10^{-9} mol L^{-1} to 1.3×10^{-7}mol. L^{-1}) on the growth, chlorophyll *a* synthesis and metal toxicity in the Chlorophyceae *Ankistrodesmus gracilis.* Algal cells were acclimated to each phosphorus concentrations prior to Cu exposure that occurred for 120 hours. Initial free copper ions (Cu^{2+}) concentrations were calculated using the chemical equilibrium model MINEQL$^+$, and the concentration that inhibited 50% of the algal population (EC_{50}) was calculated using the program ICp 2.0. The results showed higher Cu toxicity in cultures with lower P concentrations, indicating the importance of microalgae nutritional status to withstand the negative effects of the trace metal. These results indicate the importance of considering other parameters, such as the nutritional status and nutrients concentrations in the environment for a complete assessment of the toxicity of trace metals to microalgae.

Key words: Free copper; orthophosphate; acute toxicity; Chlorophyceae, eutrophication.

1 - Introduction

Phosphorus is an essential nutrient to microalgae as constituent of nucleic acids and adenosine triphosphate; acting in enzymatic synthesis and energy transfer in photosynthesis. This element can be limiting in natural freshwater aquatic environments (Cembella et al., 1982; Van Mooy et al., 2009) and in subtropical oceans gyres (Van Mooy et al., 2006), affecting physiological parameters (Napoléon et al, 2013) or acting in eutrophication (Carpenter, 2005). Microalgae may present luxurious uptake of phosphorus, e.g., absorb more phosphorus than needed for growth, and release phosphatase enzymes to release P from organic P complexes when the environment is orthophosphate-depleted (Fogg, 1973; Lai et al., 2011; Reynolds, 2006).

Phosphate in excess can be stored as polyphosphate granules that act as P reserve for the cells (Omelon and Grynpass, 2008; Rhee, 1973). Twiss and Nalewajko (1992) and Yu and Wange (2004) showed that such granules could be useful in metal detoxifying mechanisms. According to literature data, when algae are exposed to phosphorus-scarce environments, polyphosphate granules allow its regular growth and physiological processes unaffected for about 4 cell duplications (Bhola et al., 2011; Fogg, 1973; Reynolds, 2006). However, if the exposure to low P conditions is extended, then intracellular biochemical and physiological changes occur (Beardall et al., 2005; Ji and Sherrel, 2008) and, in such situation, the cells alter its phosphorus demand, requiring less P (Bertilson et al., 2003; Van Mooy et al., 2009).

Copper is essential to microalgae, supporting electron transfer in photosynthesis, enzymatic co-factor in respiration, iron metabolism and redox processes (Bossuyt and Janssen, 2004, 2005). However, concentrations above trace amounts can be toxic, decreasing growth rate, chlorophyll *a,* photosynthesis, and respiration (Baumann et al., 2009; De Schamphelaere et al., 2007; Lombardi et al., 2007; Rodgher et al., 2008; Tripathi and Gaur, 2006; Wang and Dei, 2001) or affecting cell membrane permeability (Sivakumar et al, 2010). Copper internalized can bind with specific molecules, compete with nutrients and exert toxic effects affecting cell general metabolism (Rainbow, 2002; Sivakumar et al., 2010). In the environment, Cu can be present as free ions or complexed to organic or inorganic ligands (Stumm and Morgan, 1996).

It has been shown that major nutrients such as P affect metal toxicity in microalgae and, in P depleted conditions, the toxic effects can be increased (Guasch et al, 2004; Hall et al, 1989a;

Kaneko et al, 2004; Serra et al, 2010), however the specific role of major nutrients, such as P, N and Si on trace metal uptake in microalgae is not completely known (Ji and Sherrel, 2008; Kamaya et al., 2004; Wang and Dei, 2001). Guasch et al. (2002; 2004), Ivorra et al. (2002), Luoma (1983), Riedel and Sanders (2003) and Serra et al. (2010) showed that high phosphorus concentrations in comparison with their controls has led to increasing tolerance to copper, increasing the EC_{50} value to microalgae.

The aim of the present study was to evaluate the effects of phosphorus on copper acute toxicity to the freshwater microalgae *Ankistrodesmus gracilis*. Due to the P storage capacity in microalgae and to guarantee cell metabolism was responding to the desired phosphorus (P) concentration, algal cells were previously acclimated at each specific P concentration to be tested in different combinations with Cu. The results presented are a contribution to the present knowledge of the complex interactions among phytoplankton, copper and dissolved phosphorus.

2 – Material and methods

2.1 – Algal culture

The freshwater microalgae *Ankistrodesmus gracilis* (Chlorophyceae) identified as 005 CH was obtained from the freshwater algae culture collection of the Botany Department at Federal University of São Carlos (São Carlos, SP, Brazil). Stock cultures were kept in sterile (autoclaved for 20 min at 121ºC) L.C. Oligo medium (AFNOR, 1980) at initial pH 7.0. Cultures were kept under controlled conditions of light intensity (150 µmol m^{-2} s^{-1}), photoperiod (16:8 h light: dark cycle) and temperature (22 °C ± 2).

Copper toxicity to *A. gracilis* was investigated at several different P conditions in P acclimated cells. Phosphorus (P) was furnished as K_2HPO_4 in 4 different concentrations: 2.3 x 10^{-4} – control (recommended in LC Oligo medium); 1.1 x 10^{-4}; 4.6 x 10^{-6}; 2.3 x 10^{-6}mol.L^{-1}. Microalgal cells acclimation was performed using the procedure described in Lombardi and Maldonado (2011) that is based in semi-continuous cultures. In our conditions, partial renewal of culture medium was performed every 72 h, depending on cell density, which was kept around 2 x 10^5 cells mL^{-1}. For each P concentration, cells were grown in this system for 45 days, with medium being renewed at each 3 days, in semi continuous culture system and used for the acute copper toxicity experiments after at least 3 statistically similar growth rates. In this condition, algae were considered acclimated and its metabolism reflecting the external P concentration.

Dissolved orthophosphate content in culture media was determined employing the ascorbic acid method (APHA, 1995). No copper acclimation was performed.

Aseptic conditions (UV light under a flow of filtered and sterile air) were maintained throughout and only sterile materials were used to avoid culture contamination whenever culture manipulation would be performed.

2.2 – Acute toxicity tests

Exponentially growing and P acclimated *Ankistrodesmus gracilis* cells were exposed for 120 h to different copper (Cu) concentrations, as shown in Table 1. The concentration of 0.69 x 10^{-8} $mol.L^{-1}$ is the control for copper (recommended in LC Oligo medium). Copper solutions were made by serial dilutions of $CuCl_2$ Titrisol 1000 mg L^{-1} (Merck) in ultra pure water (Barnstead Easy Pure II, Thermo Scientific, Dubuque, IA, USA). The Cu experiments were done in triplicate using 500 mL polycarbonate Erlenmeyer flasks containing 200 mL of sterile culture medium. Cells were inoculated to provide initial cell density of approximately 10^5 $cell.mL^{-1}$. All materials involved in algae maintenance and toxicity tests were previously washed with neutral detergent and kept for 7 days in 10 % HCl for metal cleaning. Free copper ions concentrations in culture media were calculated using the chemical equilibrium model MINEQL+ 4.62.3 (Environmental Research Software, Hallowell, ME, USA).

Table 1: Inorganic phosphorus (mol. L^{-1}) and free copper concentrations ($x10^{-8}$mol. L^{-1}) that *Ankistrodesmus gracilis* was exposed to. The first line in the table represents free copper ions and the first row represents the inorganic phosphorus concentrations. Crosses represent the combination phosphorus/copper used for treatments.

Cu^{2+} / PO_4	0.69	1.2	2.1	3.0	3.8	4.8	6.1	7.4	8.3	8.9	10.0	13.0
$2.3x10^{-4}$	*x*	*x*		*x*			*x*	*x*	*x*	*x*	*x*	*x*
$1.1x10^{-4}$	*x*	*x*		*x*			*x*	*x*			*x*	*x*
$4.6x10^{-6}$	*x*	*x*	*x*	*x*	*x*	*x*	*x*					
$2.3x10^{-6}$	*x*	*x*	*x*	*x*	*x*	*x*	*x*					

2.3 – Biomass

Cell densities were monitored initially and every 24 h after initial Cu exposure. Samples were fixed with acetic lugol and cells were counted under an optical microscope (Leica, DMLS) in an Improved Neubauer-Bright Line hemocytometer.

Chlorophyll *a* was determined as described by Shoaf and Lium (1976) in 10 mL samples filtered through cellulose ester membranes (0.45 µm pore size) and extracted with dimethylsulfoxide. After pigment extraction, optical density was measured in a spectrophotometer (HACH DR 5000, USA) at 664 and 647 nm wavelengths. Blanks were performed using a clean filter submitted to the same extraction procedure. Chlorophyll *a* concentration ($\mu g.mL^{-1}$) was calculated as described in Jeffrey and Humphrey, (1975) according to the following equation.

$$\text{Chl a} = (11.93 \times \lambda 664 - 1.93 \times \lambda 647) \times \frac{\text{solvent volume}}{\text{filtered volume}}$$

Where, λ664 = absorbance at 664 nm; λ647 = absorbance at 647 nm.

2.5 – Data analysis

EC_{50} was calculated using ICp 2.0 software (Environmental Protection Agency, Duluth, MI, USA) using cell density at 120 h exposure. Cell density and chlorophyll *a* data were evaluated for normality and homogeneity, ANOVA test and Tukey's post hoc ($p<0.05$), using GraphPad Instat 3.0 software.

3 – Results

According Cu speciation study approximately 12% of total added Cu remained available to the algae (Fig 1) in the control condition, while 57 – 60% were complexed with citrate, present in the citric acid and iron citrate that are part of the L.C. Oligo culture medium composition.

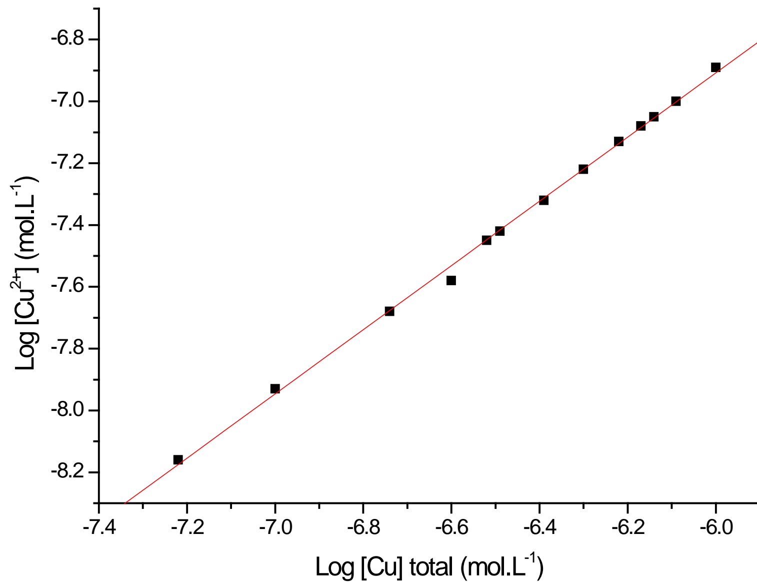

Figure 1. Relation between added copper and free copper ions (calculated using MINEQL+ v. 4.62.3) present in LC Oligo medium. Linear equation $y = -0,677 + 1,038\ x$; R = 0,99902.

EC_{50} for Cu as function of P concentrations in *Ankistrodesmus gracilis* cultures are presented in Figure 2. They varied from 4.1×10^{-8} mol. L^{-1} free Cu^{2+} ions in the treatment with 4.6×10^{-6}mol. L^{-1} P to 7.9×10^{-8}mol. L^{-1} free Cu^{2+} ions in the P control treatment (2.3×10^{-4}mol. L^{-1} P concentration). These results demonstrated that the cells responded better to excess Cu when grown in cultures with more P. The inverse was observed, P limited cells were more sensitive to Cu ions.

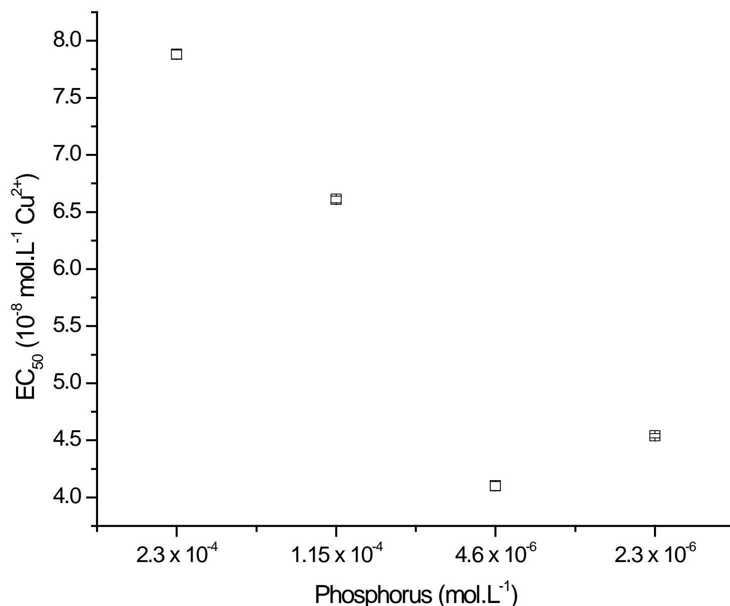

Figure 2. Inhibition concentrations to 50% (EC_{50} – 120 h exposure) of *Ankistrodesmus gracilis* cells acclimated to different phosphorus concentrations reported as function of free Cu^{2+} ions. EC_{50} values calculated using ICp 2.0 software.

Cell densities of *A. gracilis* after 120 h exposure are presented in Figure 3. It shows a general reduction in cell densities as Cu increased in culture media; the higher the Cu and the lower the P, the more drastic was cell density decrease. Growth reduction induced by Cu toxicity was dependent on P concentrations. *A. gracilis* cultures acclimated to the lowest P concentration presented the lowest growth rate when exposed to 6.1 x 10^{-8} mol. L^{-1} free Cu^{2+} concentration with the respective EC_{50} at 4 x 10^{-8} mol L^{-1}. The highest cell density was obtained for the control cultures, where the highest P and lowest Cu were added.

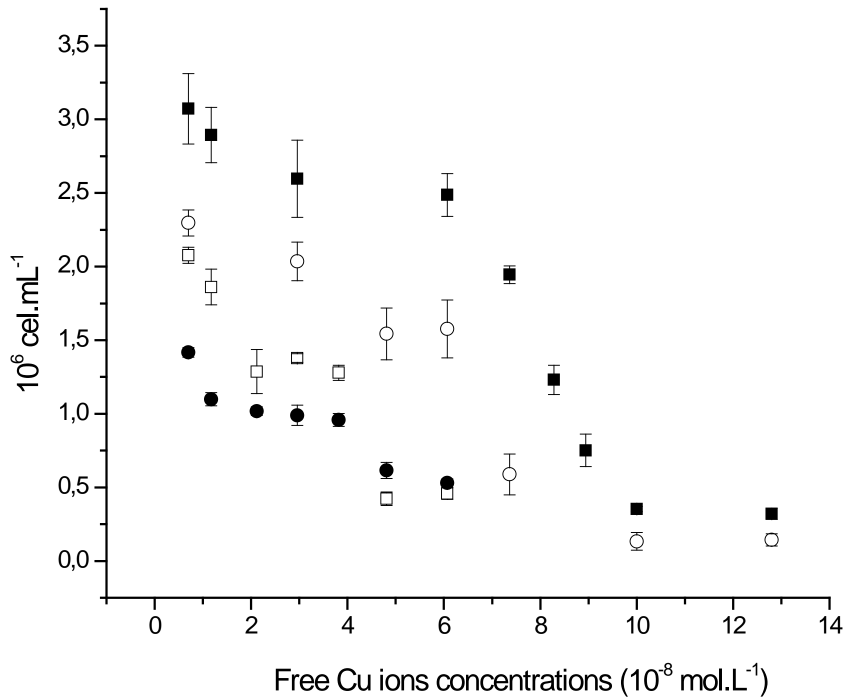

Figure 3. Cell density (cells mL^{-1}) of P acclimated *A. gracilis* at 120 h Cu exposure as function of free Cu ions (mol L^{-1}) in the medium. Symbols refer to the different phosphorus concentrations (full square: control, $2.3x10^{-4}$mol. L^{-1} P; open circle: $1.1x10^{-4}$mol. L^{-1} P; full circle: $4.6x10^{-6}$mol. L^{-1} P; open square: $2.3x10^{-6}$mol. L^{-1} P).

Chlorophyll *a* concentration (Figure 4) behaved as cell density; with a decrease in the pigment content with Cu increase in culture medium. However, an interesting fact to be observed in the Cu control (6.9 x 10^{-8}mol. L^{-1} Cu^{+2}) was an increase in chlorophyll *a* in moderate P limitation (1.1 x 10^{-4} and 4.6 x 10^{-6}mol. L^{-1} P) as compared with P control (2.3 x 10^{-4}mol. L^{-1} P) experiment.

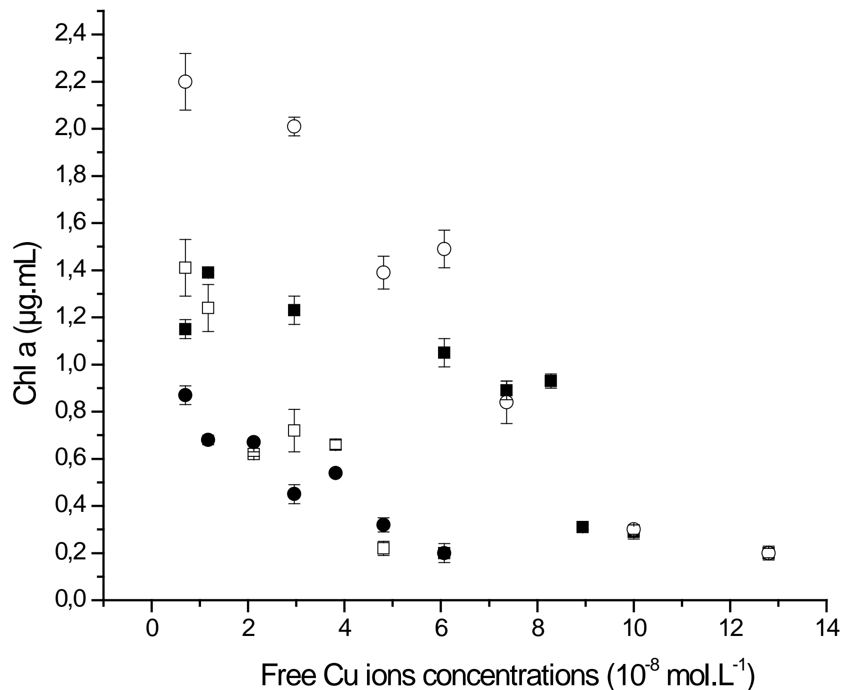

Figure 4. Chlorophyll *a* (µg.mL^{-1}) of P acclimated *A. gracilis* at 120 h Cu exposure as function of free Cu ions (mol L^{-1}) in the medium. Symbols refer to the different phosphorus concentrations (full square: control, 2.3x10^{-4}mol. L^{-1} P; open circle: 1.1x10^{-4}mol. L^{-1} P; full circle: 4.6x10^{-6}mol. L^{-1} P; open square: 2.3x10^{-6}mol. L^{-1} P).

4 – Discussion

Speciation analysis in metal ecotoxicity investigations is important because metal toxicity is related with free metal ions and not with total metal concentrations (Allen et al., 1980; Lombardi et al, 2007; Mendes et al, 2013; Sunda and Huntsman, 1998). Free ionic copper (Cu^{2+}) is one of the most toxic forms of this metal (Giesy et al., 1983; Lombardi et al., 2002; Sunda and Lewis, 1978) and it can compete with nutrients and bind with organic ligands, being more or less available to the organisms (Sunda and Huntsman, 1998). In the present research, citrate-complexed copper dominated Cu speciation in L.C. Oligo medium. Considering the variation of metal speciation with environmental conditions and culture medium composition, literature comparisons of metal toxicity data can be difficult. For example, because approximately 60% of the added Cu was of lower availability to *A. gracilis* (citrate complex) than free Cu^{2+} ions, if we

were to consider nominal Cu for EC_{50} calculations, the EC_{50} values obtained would have been super estimated, e.g., more Cu would be necessary to cause similar toxic effect. However, if the results are reported as function of free Cu ions, than such artefact does not occur. We propose that metal toxicity, particularly, trace metals such as Cu, which has high affinity for organic ligands (Tonietto et al., 2014) should be reported as function of the free metal ion. This is particularly important in eutrophic environments, where organic materials are present in significant amounts and can complex copper ions.

We showed that the presence of free Cu in concentrations higher than 2×10^{-8} mol L^{-1}, regardless of P concentration, resulted in cell density decrease after 120 h exposure. Nalewajko and Olavenson (1995) suggested that growth was a sensitive parameter to evaluate Cu toxicity under nutrient replete conditions and literature data suggest that sublethal levels of Cu can result in population density and growth rate reductions (Guanzon et al., 1994; Hall et al., 1989a, b; Stauber and Florence, 1987). Rodgher et al. (2008) found similar results for the freshwater microalgae *Pseudokirchneriella subcapitata* exposed to free Cu concentrations ranging from 1.0 $\times 10^{-10}$ to 1.3×10^{-8} mol. L^{-1}, and in the highest concentration cell density was 20% lower than the control. Lombardi and Maldonado (2011) exposed the marine microalgae *Phaeocystis cordata* to 13 different free Cu concentrations ranging from 1.4×10^{-15} to 3.1×10^{-10} mol. L^{-1} and observed reduction in growth rate and photosynthesis as Cu was increased in cultures. Similar results were found by Knauer et al. (1997) in several freshwater algal species; the authors suggested a metal intracellular immobilization mechanism. Perales-Vela et al. (2007) showed that after exposing *Scenedesmus incrassatulus* for 144 h to Cu its growth was more sensitive than chlorophyll *a* synthesis to evaluate Cu toxicity.

The present results confirmed P importance to *A. gracilis,* reducing its growth when in shortage. This confirms the results of Alcoverro et al. (2000), Beardal et al. (2001), Bhola et al. (2011), Lai et al (2011), Rhee (1973), Spijkerman and Wacker (2011). Besides reducing growth, we showed that P affected Cu tolerance and toxicity to *A. gracilis*. Phosphorus replenished cells had higher density after 120 h Cu exposure and exhibited higher Cu tolerance than P limited cells. The interaction effects between P and Cu are illustrated by the EC_{50} decrease as P decreased in culture media, which supports the hypothesis that P-depleted cells are in general more sensitive to Cu. This confirms the results presented in Hashemi et al. (1994), Rijstenbil et al. (1998), Takamura et al. (1990), and Twiss and Nalewajko (1992). Chen (1994) found a strong

correlation between EC_{50} for Hg and P concentrations in culture media, suggesting that EC_{50} was dependent on the nutrient level. Our results showed similar behaviour for Cu instead of Hg. We showed that EC_{50} value from treatment with 2% of the P present in the controls were around 47% lower than the control, demonstrating higher sensitivity to Cu in P limited conditions. Hall et al. (1989a) obtained higher Cu sensitivity and intracellular metal increase in P limited *Chlorella vulgaris* and *Chlamydomonas geitleri*; Serra et al. (2010), which investigated Cu toxicity to the diatom *Nitzchia perminuta* in different P concentrations (replete and depleted), observed that P-depleted cultures were 1.5 to 2 times more sensitive to Cu than P-replete cultures. In their experiment, the authors added P to P-depleted cultures and observed 1.6 times reduction in Cu toxicity after P addition.

The decrease in Cu tolerance in P-limited *A. gracilis* can indicate problems in cell membrane permeability, which may be due to a reduction in membrane lipids, such as phospholipids, as observed in Lombardi and Wangersky (1991). These authors found less phospholipids in P-limited *Chaetoceros gracilis* in comparison with controls and N-limited cells. Literature data show a substitution of phospholipids in cell membranes for sulfolipids or galactolipids in P depleted conditions (Andersson et al., 2003; Van Mooy et al., 2006; Van Mooy et al., 2009). Hall et al. (1989a) assumed that increased Cu internalization in *Chlorella vulgaris* occurred due to higher cell permeability under P-limitation, which could facilitate the entrance of ions and other compounds into the cell, damaging cell structure and function. This is supported by the results of Kamaya et al. (2004), which evaluated the effects of P reduction in culture medium and Cu, Zn, polyphenols and herbicides toxicity to *P. subcapitata,* and found that P limitation influenced algal sensitivity to all toxicants, not only the metals.

Besides population growth and cell density, Cu affected the synthesis of chlorophyll a in *A. gracilis*. A maximum 80% reduction in chlorophyll *a* was obtained in the highest Cu concentration regardless of P concentrations. This is in accordance with the results of Perez et al. (2006), which obtained chlorophyll *a* reduction in phytoplankton exposed to different Cu concentrations. Considering chlorophyll *a* synthesis, *A. gracilis* was more sensitive to Cu than other microalgae, as reported in Rodgher et al. (2008), and Tripathi and Gaur (2006). Rodgher et al. (2008) exposed *Pseudokirchneriella subcapitata* to different concentrations of Cu and observed a reduction in chlorophyll *a*. These authors showed 12 times reduction of the pigment in 1.3×10^{-7} mol. L^{-1} Cu^{2+} concentration in comparison with the control. Tripathi and Gaur

(2006) observed chlorophyll *a* and growth rate reductions in *Scenedesmus* sp. exposed to 2.5 x 10^{-6} mol. L^{-1} for 48 h. According to Kupper et al (1996), chlorophyll *a* reduction can be due to replacement of Mg ions by metallic ions with the same charge (2+), such as Cd, Cu, Zn, Ni and Pb, causing an important mechanical damage to the pigment, affecting the electron transfer in photosynthesis.

Acclimating the algae to specific P concentrations used has guaranteed that cells physiology were in fact behaving according to the external P level, supporting the good relation we obtained between P concentrations and Cu toxicity. It is known that phytoplankton adjust their uptake machinery for changes in external limiting nutrient for a better use of the resources and maintain the maximum growth rate possible, so possibly reducing damages caused by the nutrient limitation (Bonachela et al, 2011; Geider et al, 1998; Morel, 1987; Smith and Yamanako, 2007). Twiss and Nalewajko (1992) studied *Scenedesmus acutus* in P-deficient conditions, in which the algae remained for no more than 2 or 3 days. The authors concluded that such short period of time (2 to 3 days) was not enough to affect the cell physiology. Based on literature, we agree with Cembella et al (1984) and Madariaga and Joint (1992) about the importance of phytoplankton being to exposed to the new environmental conditions for a time long enough that adaptation and steady-state conditions are achieved, as performed in the present study.

5 – Conclusions

According to our results, *Ankistrodesmus gracilis* was a sensitive organism to copper ions, and phosphorus availability was an important factor affecting such sensitivity and, consequently the toxicity of the metal. In lower phosphorus concentrations, P acclimated algae were less tolerant to copper, demonstrating the importance of nutritional status of the cells to handle with the toxicant.

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CAPÍTULO 2

Um estudo comparativo da composição bioquímica de três espécies de microcrustáceos utilizados como alimento vivo em aquicultura

A larvicultura é hoje o principal entrave da aquicultura mundial. A disponibilidade de alimento vivo é essencial para o desenvolvimento de fases jovens de peixes e crustáceos. A qualidade do alimento ingerido pelas larvas desses organismos tem impacto direto em suas taxas de crescimento, reprodução e longevidade. O zooplâncton é muito utilizado como alimento vivo na aquicultura em virtude de seu alto valor nutricional, sendo uma excelente fonte de proteínas, lipídios e minerais. O Anostraca *Artemia* sp. há anos vem sendo utilizadona alimentação de peixes e crustáceos cultivados. No entanto, seu custo de produção tem sido cada vez mais elevado, especialmente em sistemas de cultivo de espécies de água doce, já que esse animal é originário de águas salobras. O Anostraca neotropical dulcícola *Dendrocephalus brasiliensis,* popularmente conhecido como branconeta, tem grande potencial para utilização na aquicultura nacional em virtude de seu tamanho (25mm), facilidade de cultivo e grande atratividade para os peixes. Tal espécie pode ser uma boa alternativa de substituição da *Artemia* sp., implicando em significativa redução dos custos de produção. Embora protocolos de cultivo de *D. brasiliensis* já tenham sido desenvolvidos com elevada eficiência de produção, e várias espécies de peixes tropicais tenham alta aceitação desse alimento (Projeto Temático FAPESP 2008/02078-9), ainda existem poucas informações disponíveis sobre sua composição bioquímica e valores nutricionais.

Assim, visando ampliar o conhecimento sobre a qualidade de *D. brasiliensis* como alimento vivo, o segundo capítulo desta tese corresponde a um estudo da composição bioquímica dessa espécie comparativamente a outras duas espécies de microcrustáceos normalmente utilizados como alimento de larvas de peixes e crustáceos em sistemas aquícolas. O estudo apresentado neste capítulo é o seguinte:

2.1 Comparative biochemical composition of two anostraceans (Dendrocephalus brasiliensis and, Artemia sp.) and one cladoceran (Daphnia magna) species.

Comparative biochemical composition of two anostraceans (*Dendrocephalus brasiliensis* and *Artemia* sp.) and one cladoceran (*Daphnia magna*) used as live food in aquaculture

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Abstract

In aquaculture, first feeding is an important issue because it can affect all the fish development. Usually, *Artemia* and rotifers are used as live food, especially in marine aquaculture, but other alternatives must be found. The aim of this study was to evaluate biochemical composition of a Brazilian native fairy shrimp (*Dendrocephalus brasiliensis)* compared to *Artemia* sp. and *Daphnia magna*, frequently used in aquaculture. Results of protein, carbohydrate and lipids revealed that the fairy shrimp contain significant amounts of protein (around 1.7 mg per adult) and carbohydrates (around 150 µg per adult). Their lipid composition, especially phospholipids, varied according to the age and sex: phospholipids concentrations were bigger in younger females when compared with males, but these values changed in older animals, when males presented higher amounts of phospholipids. So, *D. brasiliensis*seems to be a good option to provide satisfactory energy to the animals that use this food source. We observed differences in carbohydrate values in *Artemia* males (51 ± 12 µg per adult) and females (179 ± 3 µg per adult) with same age and almost same length. *Daphnia magna* presented a lipid profile with high percentage of triacylglycerol, that could represent a good source of omega-3, but the amounts of carbohydrates (30.3 ± 0.4 $\mu g.ind^{-1}$) and proteins (11. 8 ± 1.5 $\mu g.ind^{-1}$) were low. According to the results obtained, we conclude that *D. brasiliensis* is a good quality food and has the potential to be used in aquaculture industry.

Keywords: fairy shrimp; lipid classes, protein, carbohydrate

1 Introduction

Aquaculture is an important industry, providing alternatives sources of food, searching for to achieve the best results, close or better than in natural environments. Based on this, a high number of studies are conducted aiming the evaluation of using different organism as live food and different enrichments, trying to obtain the best relation cost/benefit.

Zooplankton is known as a good source of energy for fish, especially in relation to lipid content (wax esters and fatty acid composition) (Houlihan et al., 2001; Olsen et al., 1991).Usually, *Artemia* spp and rotifers have been used as food, especially with marine fish, but other natural or artificial food sources must be studied, providing a higher number of options to furnish the best results, according to the species of interest in aquaculture.

The freshwater Brazilian native Anostraca *Dendrocephalus brasiliensis* Pesta 1921 (Crustacea: Anostraca, Thamnocephalidae), popularly called “branconeta”, lives in temporary freshwater ponds and presents morphological and reproductive system similar to *Artemia*. Its geographical distribution is between Argentina and Brazilian State of Piauí (César, 1989; Calviño and Petracini, 2004), being registered naturally in different Brazilian States (Pesta, 1921; Lutz, 1929; Lopes, 2007; Passos, 2012) or as exotic species (Mai et al, 2008). It is a generalist filtering species, feeding on suspended material, such as bacteria and rests of organic matter (Calviño and Petracini, 2004), but apparently prefers phytoplankton (Lopes, 2002). Their natural predators are larvae and adult insects, and fish that live in temporary ponds (Coelho and Araújo, 1982). Although it has been presented good results as feed to some fish species and the shrimp *Litopenaeus vannamei* (Yflaar, 2003; Lopes, 2007), researches about biological and ecological processes of *D. brasiliensis* are in initial phase. Their characteristics, such as good size after hatching, easily captured by fish larvae and easy culture in tanks (Lopes, 2002), make this species an interesting object of study to alternative use as live food in aquaculture, especially in freshwater.

Related to lipid classes, *Artemia* cysts have high EPA (20:5ω3) levels (Olsen et al, 1999); however nauplii have low EPA and DHA (22:6ω3) levels (Navarro et al, 1999). These animals are not able to synthesize DHA (Estevez et al, 1998), metabolizing it faster than the other fatty acids (Olsen et al, 1997). So, to reach best nutritional value, it is necessary to enrich them, which makes their use costly. Zooplankton biochemical composition can change according to ingested

food, being manipulated in culture systems (Pond and Tarling, 2011; Rainuzzo et al., 1994; Støttrup et al, 1999), but it can decrease the cost / benefit of its use.

The aim of this study was to evaluate the biochemical composition (protein, carbohydrate, lipid and lipid classes) of *D. brasiliensis* compared to two species often used as live food in the fish and crustacean cultures, especially in the first developmental stages, where occurs the biggest loss in the aquaculture process. The main purpose is to evaluate whether branconeta have sufficient nutritional quality to be used as live food in aquaculture systems and may be a cheaper alternative, especially in poor countries.

2 -Material and methods

2.1 - Organisms cultures

2.1.1 - *Artemia* sp.

Artemia sp. cysts were hatched at 27 ºC, and cultivated at 5,000 L tanks with 30‰ salinity. Initially, yeast was provided as food source and after the natural establishment of different algae, no more food was provided to animals.

2.1.2 - *Daphnia magna*

Daphnia magna was cultivated in 10,000L tanks with algae community previously established. Phytoplankton used as food source was composed of a mixture of different species from natural environments in São Paulo State, Brazil. Once algae community is established, we did not have to provide any other food source to the animals.

2.1.3 - *Dendrocephalus brasiliensis*

Cysts of *D. brasiliensis* were dried for 3 months and then hatched in 10,000 L tanksin the Experimental Aquaculture Station of the Federal University of São Carlos. Some of these cysts were collected and hatched in the lab, in 50 L aquariums, containing around 20 liters of water from fish tanks and 20 liters of dechlorinated tap water. Cysts were hatch at 22ºC and then temperature was increased stepwise for12 hours until it reached 28ºC. Organisms were fed with a

mix of different algae species, especially *Ankistrodesmus gracilis* and *Chlorella* sp., until biochemical analyses (~3 weeks).

2.2 – Biochemical analysis

All glassware were first washed with tap water and neutral detergent, then rinsed with tap water and placed in10% hydrochloric acid for 7 days to decontamination. Glass fiber filters (GF/C; Boeco, Germany) were burned (400°C for12h) and used to retain organisms for biochemical determinations. All material used in lipid analysis was burned (400°C for 24h) and the glassware was rinsed with methanol and chloroform prior to the use. Animals were collected in the aquarium using a plankton mesh (68 µm pore size) and transferred to a beaker.

2.2.1 – Protein

Animals were grinded and centrifuged at 4400 rpm for 15 minutes (Eppendorf 5702R, Germany) with 1.5 mL of NaOH 0.5N and the proteins were extracted in an oven at 100°C for 1 hour. After this time, samples were centrifuged at 4400 for 15 minutes and the supernatant was transferred to another vial, then protein reagent (0.01% Coomassie Blue, 4.7% ethanol and 8.5% phosphoric acid) was added and the absorbance at 595 nm was measured (HACH DR 5000; HACH Company, USA). Proteins were quantified according to Bradford (1976) method, using a calibration curve with bovine serum albumin as standard.

2.2.2 – Carbohydrate

Total intracellular carbohydrates were performed using the modified phenol-sulphuric method according to Liu et al. (1973). Organisms were grinded and centrifuged at 1500 rpm for 10 minutes (Eppendorf 5702R, Germany), the supernatant was discarded and the pellet resuspended in 1mL distilled water, 1 mL of phenol 10%, vortexed and 5 mL H_2SO_4 was added quickly to the solution and left stand at room temperature for 10 minutes, then the sample was centrifuged at 4400 rpm for 10 minutes and the supernatant read at 485 nm against a blank reagent and quantified with calibration curves using glucose as standard.

2.2.3 – Lipids

Total lipids and lipid classes were performed through thin layer chromatography with flame ionization detection (TLC/FID) using anIatroscanMK6 (Mitsubishi Kagaku Iatron Inc., Tokyo, Japan) according to the methodology described in Parrish (1987; 1999) that are based on Folch et al (1957). Filters were grinded with glass rod and the extraction was done using chloroform: methanol: chloroform extracted water (2:1:1), sonicated for 4 minutes (Unique Group, Indaiatuba, Brazil) and centrifuged for 2 minutes at 3000 rpm (Eppendorf 5702R, Germany). Organic layer containing lipids was removed and transferred to a lipid clean vial, then 6 mL chloroform were added and the procedure repeated for 3 times. Samples were concentrated under ultrapure N_2, sealed and stored at -20°C until analysis. For the chromatography (TLC/FID), the samples were spotted onto quartz rods (Chromarod SIII) using a Hamilton syringe. Samples were focused twice in 100% acetone and placed in a constant humidity chamber for 5 minutes. Three solvent systems were used for the complete sample development that resulted in the detection of 8 lipid classes. The first solvent system was composed of hexane: diethyl ether: formic acid (98.95:1:0.05); the second was hexane: diethyl ether: formic acid (79:20:1); and the third was chloroform: methanol: chloroform extracted water (5:4:1). After each development, the rods were kept in the Iatroscan for 5 minutes before scanning and for 5 minutes in the humidity chamber after scanning. Lipid classes were identified from calibration curves made with lipid standards obtained from Sigma-Aldrich (USA). The analytical conditions for the TLC-FID runs were: hydrogen flow 173 mL min^{-1}, air flow 2 L min^{-1} and scan speed 4 mm s^{-1}.

2.3 – Data analysis

Protein, carbohydrates and lipids data were submitted to normality and homogeneity analysis, ANOVA test and Tukey's post hoc ($p<0.05$), using GraphPad Instat 3.0 software.

3 - Results and Discussion

Our results suggest that, related to biochemical composition, especially protein content, *D. brasiliensis* could be a good option to be used as live food to cultured fish and crustaceans.

In Figure 1 we compare the protein content of the three studied species: *D. brasiliensis, Artemia* sp. and *D. magna*.

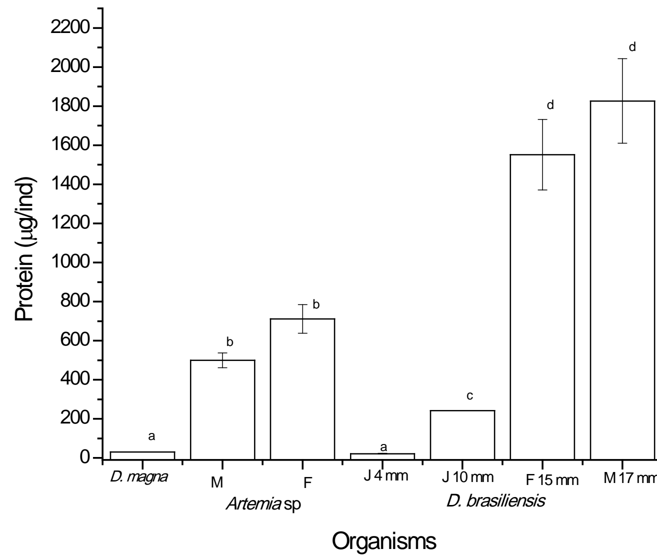

Figure 1: Protein values (µg.ind^{-1}) of different zooplankton species. M = male; F = female; J = juvenile. Values represent mean of n=3 ± SD

The results of the present work show that bigger and older *D. brasiliensis* had highest values of protein contents: more than twice those obtained in *Artemia* and around 9 times bigger than those observed in juveniles. This high amount of protein in bigger *D. brasiliensis* is an important fact regarding its nutritional value, since amino acids, especially in the free form, can provide another valuable source of energy. Generally, the first energy sources are carbohydrates and lipids, but if the food is rich in free amino acids, these can provide carbon skeletons that form new carbohydrates and lipids (Evans et al., 2000).

Carbohydrate values of *Daphnia magna, Artemia* sp and *Dendrocephalus brasiliensis* are shown in Figure 2.

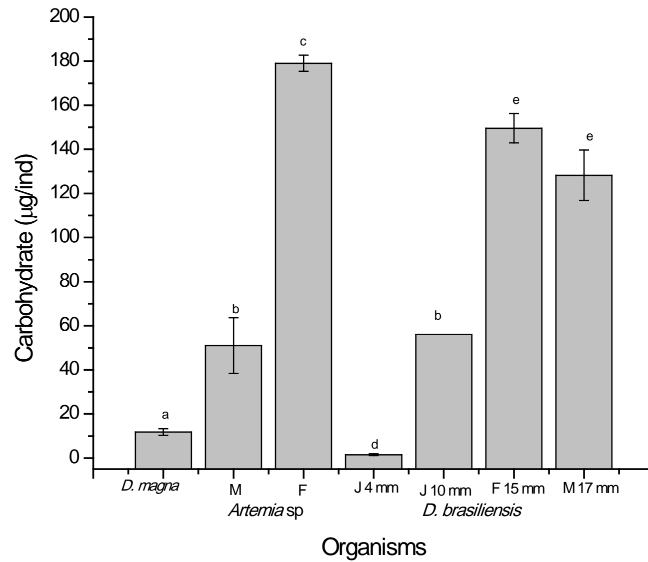

Figure 2: Carbohydrate values (µg.ind^{-1}) of different zooplankton species. M = male; F = female; J = juvenile. Values represent mean of n=3 ± SD

Analyzing the carbohydrate values in different organisms, we observed that *Artemia* female show the highest value, almost 3 times higher than male *Artemia* with same size and age, and almost 10 times higher than *Daphnia magna*. It could be observed an increase in carbohydrate content in *D. brasiliensis* with the age, reaching the highest value in female around 15 mm length, but in juvenile phase (~ 4 mm), the carbohydrate value is lower than *Daphnia magna.* While *D. brasiliensis* shows higher protein, female *Artemia* is the best option if the aim is a diet carbohydrate rich.

Lipids are carbon-rich molecules of high-energy value, being important storage sources to marine and freshwater zooplankton. Microalgae are primary producers of lipids and zooplankton acts as a link between producers and higher trophic levels. Lipid reserve represents the integration between acquisition by food and losses by respiration and reproduction (Vanderploeg et al, 1992), being influenced by environmental conditions, such as nutrient concentrations (Breteler et al, 2005). Some lipid constituents are essential nutrients to the animals, such as polyunsaturated fatty acids (Parrish et al, 2005).

Lipid classes of different stages of *D. brasiliensis* are presented in figure 3, and lipid classes of *Daphnia magna* and *Artemia* sp are shown in Figure 4.

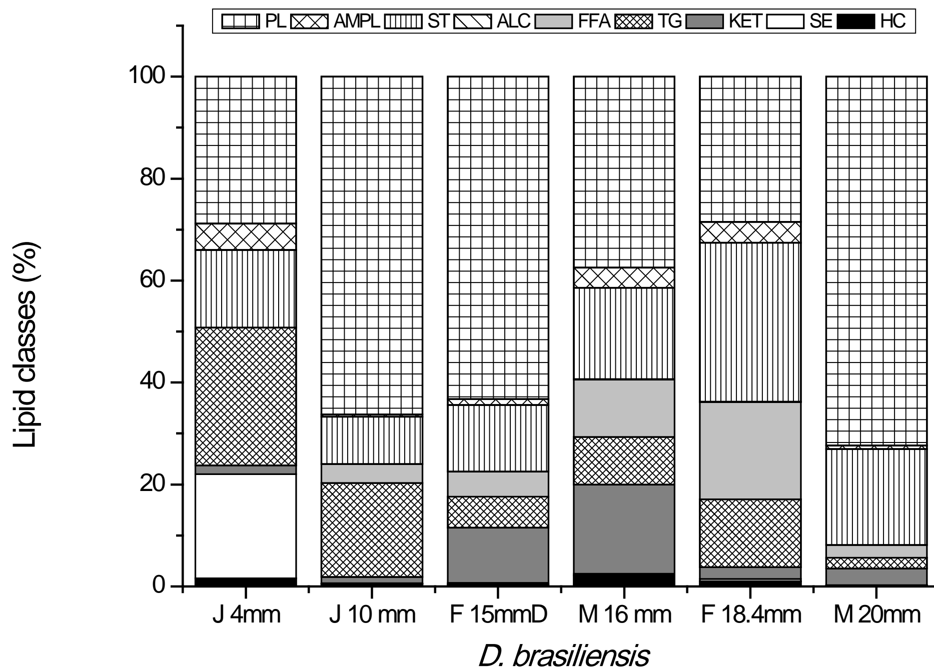

Figure 3: Lipid classes of different stages of *D. brasiliensis*. J = juvenile; M = male. F = Female.

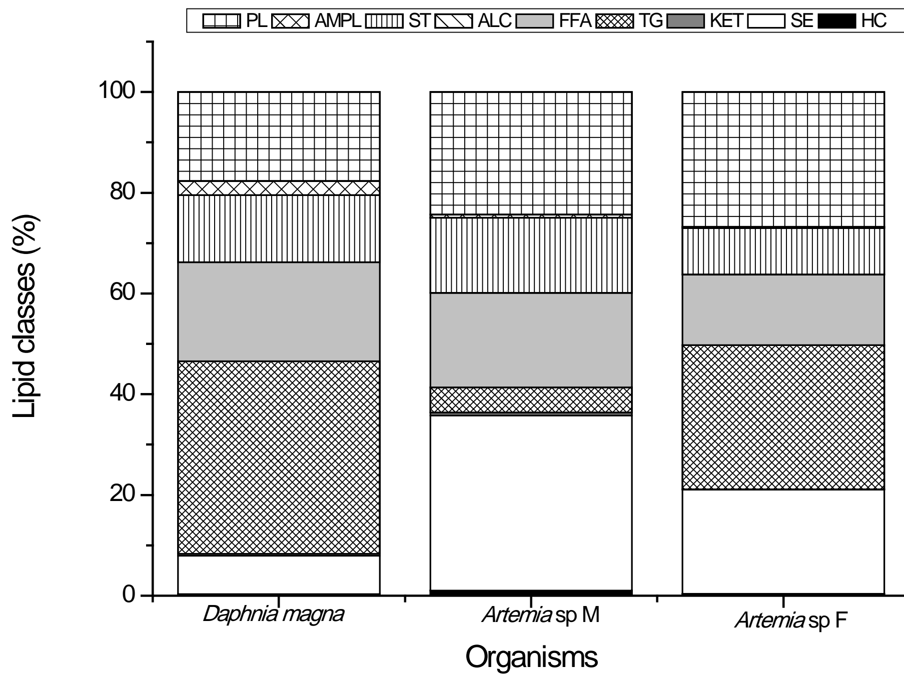

Figure 4: Lipid classes of different stages of *D. brasiliensis*. J = juvenile; M = male. F = Female.

Comparing the lipid classes in *D. brasiliensis*, we can observe a variation in composition according to the stage and sex. In juveniles around 4 mm, there is a high amount of wax ester (SE), the highest contribution of this class is on this stage, but lower than SE contribution in copepods (Conelly et al., 2012). When the juveniles are around 10 mm, there is a change in the pattern, with much more phospholipids (PL) and less sterol (ST), SE and triacylglycerol (TG). With 15 mm, the females present high amounts of PL and low TG, and this is different from the males that have almost the same length, and have lower amounts of PL, but higher amounts of ketone (KET), TG, free fatty acids (FFA) and ST. When the females are older, the lipid class's composition change, increasing the ST, FFA and TG contribution, decreasing PL content. On the other hand, older males present higher amounts of PL than young males. Based on the results, the contribution of TG in *D. brasiliensis* is smaller than in marine amphipod (Conelly et al., 2012). These differences in lipid composition are important to evaluate the best stage to offer *D. brasiliensis* as live feed *Daphnia magna* has the highest amount of TG in the organisms studied, while *Artemia* male has the highest amount of SE.

Analyzing lipid composition of the three studied species, we can observe significant differences in classes' composition according to development stage, sex and individual. Depending on the aim, one or another can be chosen to be used as live food, which means that, depending on the nutritional need of the species of interest in aquaculture, there are different options, i.e., if the species to be cultivated require more proteins, the branconeta is a best option, while *Artemia* female is a better source of carbohydrates. Fatty acids play important and diversified functions in organisms, being required in different amounts by the vertebrates (Sargent et al., 1995) and can be used as trophic marker, because of their non-degradable nature (Arts et al., 2001; Budge and Parrish, 1998; Iverson et al., 2004; Kainz et al., 2002). Some polyunsaturated fatty acids (PUFA) are named essential fatty acids (EFA) due to their requirement by the organisms to correct (Sargent et al., 1995) and healthy development (Parrish, 2009), it is recommended an evaluation of fatty acid profiles to check how is the best option to use in aquaculture and if *D. brasiliensis* needs an enrichment as rotifer and *Artemia* or can be used as copepods, without any kind of enrichment.

4 - Conclusion

Based on our results, we believe that *Dendrocephalus brasiliensis* present a great potential to be used in aquaculture, providing high amounts of protein and carbohydrate.

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CAPÍTULO 3

Um estudo sobre a influência de diferentes alimentos vivos (zooplâncton natural, rotíferos e *Artemia* enriquecidos)na composição lipídica e de aminoácidos do bacalhau do Atlântico (*Gadus morhua*).

No terceiro capítulo, buscou-se compreender as respostas bioquímicas do bacalhau do Atlântico (*Gadus morhua*) a diferentes tipos de alimento vivo. Com a oportunidade da realização do Doutorado Sanduíche no Canadá, foi possível realizar experimentos com organismos marinhos, que são melhor conhecidos que os organismos de água doce, especialmente quanto à composição lipídica. O objetivo foi avaliar a influência de diferentes organismos (naturais ou enriquecidos) na composição lipídica e de amino ácidos do bacalhau. Os experimentos foram realizados usando-se protocolos estabelecidos para o cultivo dessa espécie, com o fornecimento de rotíferos (*Brachionus plicatilis*), *Artemia* sp. e zooplâncton como itens alimentares. Neste estudo, foram determinadas as composições lipídicas e de amino ácidos (livres ou totais) nos itens alimentares e no peixe.

O estudo apresentado neste capítulo é o seguinte:

3.1 Effects of wild zooplankton, enriched rotifers and Artemia spin the Atlantic cod (Gadus morhua) biochemical composition

Effects of wild zooplankton, enriched rotifers and *Artemia*sp in the Atlantic cod (*Gadus morhua*) biochemical composition

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Abstract

The initial food can affect fish development, pigmentation, and growth. Live food seems to be the best option to achieve all those needs. Wild zooplankton and enriched rotifers and *Artemia* are usually used as food in the first stages of larvae. The present study aimed at evaluating the effects of different combinations of enrichments of rotifers and *Artemia* and the addition of wild zooplankton in the culture of Atlantic cod (*Gadus morhua*). In the diets, we observed that wild zooplankton presents better values of essential and omega-3 fatty acids when compared to enriched rotifers and *Artemia*. This better value reflects on fish development, with larvae fed zooplankton as complement to rotifers and *Artemia* growing faster than those fed without this supplement. Based on these results, we recommend the inclusion of wild zooplankton in the initial feeding of Atlantic cod larvae.

Keywords: aquaculture, fatty acids, lipids, enriched organisms

1 Introduction

In natural environments, fish can balance their diets capturing the food that is better for their needs and nutritional deficiencies in these animals are rare(Hamre, 2006). Natural fish food has high energetic value, with high levels of proteins, minerals and vitamins. In artificial environments, the organisms can be enriched with vitamins and essential fatty acids to improve the nutritional value and provide the transfer of better quality elements in the trophic chain (Coutteau and Sorgeloos, 1997).

The quality and quantity of suitable food in the first days of larval fish influence the growth rate, sexual maturity and life time(Qin et al., 1997). The best option for initial larval fish

nutrition is live food due to essential fatty acid contents (Watanabe et al., 1984). The initial diet should contain high levels of free amino acids, enzymes and water (Lazzaro, 1987) and zooplankton fulfill these requirements (Hebert, 1978).

Zooplankton, wild or cultured, have a good nutritional value as a source of proteins and a balance of amino acids, minerals and lipids. Among the lipids, the wax esters are the main storage reserve in zooplanktonic crustaceans and an important energy source for fish (Houlihan et al., 2001; Olsen et al., 1991). Their biochemical composition is altered according to ingested food (Brown et al., 1997; Nanton and Castell, 1999; Payne et al., 1998; Pond and Tarling, 2011; Rainuzzo et al., 1994) and can be manipulated in culture systems (Payne et al., 1998; Støttrup et al, 1999).

Rotifer and *Artemia* enrichments are made aiming at a nutritional composition similar to wild zooplankton (Evjemo and Olsen, 1997), this being the desired composition important to larvae success (Bell et al., 2003; Watanabe, 1993). The copepods present better nutritional composition when compared to rotifers and *Artemia* (McEvoy et al., 1998; Støttrup and Norsker, 1997) and high levels of vitamins C and E (Van der Meeren et al., 2008). In copepods, lipid composition can vary according to depth and pressure (Pond and Tarling, 2011)or due to seasonal variations(Clark et al., 2012). Their fatty acid composition can be related to their food source (Clark et al., 2012; Payne et al., 1998; Sekino et al., 1997;Støttrup and Jenssen, 1990). Due to their capacity to incorporate, modify and synthesize fatty acids, these animals maintain good DHA/EPA ratios (Drillet et al., 2011; Nanton and Castell, 1998, 1999) and can be used as trophic markers (Parrish et al., 2000). They can retain DHA to increase membrane fluidity, maintaining their activity during winter (Smyntek et al., 2008).

The aim of this investigation was to determine the influence of different foods (wild zooplankton, enriched rotifers and *Artemia*) on cod (*Gadus morhua*) cultured in tanks. Different combinations of food offerings were provided and the organisms, zooplankton and larvae, were analysed for lipid contents (lipid classes and fatty acids) and amino acid contents (total and free).

2 Material and methods

2.1 Organisms Culture

Rotifers (*Brachionus plicatilis)*, *Artemia* and cod larvae (*Gadus morhua*) were cultured in the Dr. Joe Brown Aquatic Research Building (JBARB), at the Ocean Sciences Centre (OSC), St John's, NL, Canada. The zooplankton samples were collected in nearby Conception Bay, NL, Canada.

Rotifers were cultured at 27°C and 30‰ salinity. Unenriched rotifers were fed Ori-Culture (Skretting, Vancouver, CA - 0.2-0.35 grams million^{-1}) via a peristaltic pump that dripped for 20-24 hours. The rotifers were enriched with Ori-Green (Skretting, Vancouver, CA - 0.25g million^{-1}) or with Protein Hydrolysate powder (Pollock, Ice Protein Ltd, Iceland - 0.1g L^{-1}) for 2 hours prior to being offered as live feed to the cod larvae.

Artemia were bought in 425g cans, hatched at 27°C, cultured at 24°C and 30‰ salinity. Unenriched *Artemia* were fed Ori-Culture (0.3 g million^{-1}) by a peristaltic pump that dripped for 8 hours. *Artemia* were enriched with Ori-Green (0.8 g million^{-1}) for 12 hours or Protein Hydrolysate powder (0.1g. L^{-1}) for 2 hours prior to being offered as live feed to the cod larvae.

Zooplankton was collected by towing 100µm mesh plankton net with a 1 m diameter mouth behind the OSC's Boston Whaler. After collection, zooplankton was kept in an aerated cooler and in the lab they were passed through a 400µm mesh filter and counted. Copepods (*Tempora* sp., *Oithona* sp. and *Pseudocalanus* sp.)dominated the zooplankton samples and they were fed to the cod larvae once per day in the first week (2-9 dph) and twice a day until 30 dph, representing 5-10% of the total prey available. The larvae fed exclusively on zooplankton before addition of rotifers or *Artemia* to the tanks.

The cod eggs were collected, disinfected with ozone and placed in two incubators at 6-7°C. The newly-hatched larvae were transferred to 16 tanks (400 L, each), at a density of 50 larvae. L^{-1} and exposed to 24 hour light. The water temperature was increased from 6-7°C to 10.5°C in a 10 day period. These tanks were divided into 3 treatments. Between 2dph until 9mm length (25 dph), the larvae were fed rotifers; between 9 and 13 mm length (35 dph) they fed *Artemia*.

In treatment 1 (RA - 6 tanks), the larvae were fed 3 times/day with Ori-Green enriched rotifers and *Artemia*. In treatment 2 (RA-PH - 6 tanks), the larvae were fed Ori-Green enriched rotifers and *Artemia* four times per week and Protein Hydrolysate enriched rotifers and *Artemia* 3 times per week. In treatment 3 (RA-Zoo - 4 tanks), the larvae fed wild zooplankton

(~250,000/tank) and Ori-Green enriched rotifers and *Artemia* until 30 dph (see zooplankton culture).

2.2 Sampling

For the lipid, free and total amino acids analyses the same amount of sample was filtered for rotifers, *Artemia* and zooplankton, and the same number of larvae were collected. Lipid samples were placed in 50 mL tubes which had been cleaned lipid with methanol and chloroform, and 8 mL of chloroform was added The tube tops were flushed with nitrogen, capped and sealed with Teflon® tape. Amino acid samples (total and free) were placed in 20 mL scintillation vials, previously burned at 400°C for 24 hours. All samples were stored at -20°C until the extraction and analyses.

Artemia and rotifers (unenriched, Ori-Green and Protein Hydrolysate enriched) were filtered for biochemical analysis. Zooplankton samples were homogenized and 50 mL were filtered into glass fiber filters (GF/C). All samples had two replicates and one blank.

The cod larvae were taken at 0 dph (150 larvae) and at different lengths (9, 11 and 13 mm, between 25 and 35 dph). At 9 mm length, 50 larvae per tank were collected, while at 11 and 13 mm length 30 larvae/tank were collected.

2.3 Biochemical composition

2.3.1 *Lipids*

Lipid extractions were done according to Parrish (1999) based on Folch et al. (1957) methodology, using methanol and chloroform. Methanol (4 mL) was added to the samples, which were ground with a metal rod. After homogenizing, 4 mL of chloroform: methanol (2:1)were added then 2 ml of chloroform-extracted water. The samples were sonicated for 4 minutes in an ice bath and centrifuged at 5000 rpm for 2 minutes. After centrifugation, the sample had two layers: the upper containing methanol, water and non-lipid material and the bottom containing chloroform and lipids. The bottom layer was removed using a double pipetting technique and replaced in a lipid-cleaned vial. After removing the entire bottom layer, 12 mL of chloroform were added and the samples were sonicated and centrifuged and the bottom

layer was removed. This was repeated 3more times. The entire bottom layers were placed together and were evaporated using a rotary vapor (Buchi Rotavapor R, Buchi Labortechnik AG, Switzerland), they were concentrated under nitrogen until a known volume, capped, sealed with Teflon® tape and stored at -20°C until analysis. All glassware and Teflon liners used in lipid extraction and storage were rinsed 3 times with methanol and 3 times with chloroform before use.

2.3.1.1 *Lipid classes*

Lipid classes were analyzed according to Parrish (1987; 1999), using thin layer chromatography with flame injection detection (TLC/FID) in an Iatroscan MK 6 (Mitsubishi Kagaku Iatron Inc., Tokyo, Japan). The chromatograms were transferred to the computer with the Peak Simple Chromatography Data System (SRI Model 202, 4-Channel Serial Port, SRI Inc) and analyzed with Peak Simple software (version 3.93 64 bits; SRI Inc). The data obtained were compared with calibration curves for each lipid class, using standards (Sigma-Aldrich). Air and hydrogen flow (20 and 195 mL.min^{-1}, respectively) and scan speed (3.3 mm.sec^{-1}) were kept constant during chromatographic analysis.

The sample was spotted on quartz rods covered with silica (Chromarod SIII) using a Hamilton syringe. The samples were focused in acetone 100% twice to form a band of lipid material at the origin and the rods were placed in humidity chamber for 5 minutes. The first solvent system was hexane: diethyl ether: formic acid (98.95:1:0.05) and the samples were developed for 25 and the 20 minutes, with 5 minutes in the humidity chamber between the two developments. The second solvent system was hexane: diethyl ether: formic acid (79:20:1) and the samples were developed for 40 minutes. The samples were developed twice for 15 minutes in 100%acetone, placed in the humidity chamber for 5 minutes and developed twice for 10 minutes in chloroform: methanol: chloroform extracted water (5:4:1). After each development the rods were held in the Iatroscan for 5 minutes before scanning and for 5 minutes in the humidity chamber after scanning.

2.3.1.2 *Fatty Acid Methyl Esters (FAMEs)*

FAME derivatives were prepared using14% boron trifluoride methanol (BF_3/CH_3OH) and hexane (Budge and Parrish, 2003; Morrison and Smith, 1964). The lipid extract was dried under nitrogen and 0.5 mL hexane and 1.5 mL 14%BF_3/CH_3OH added. The mixture was shaken, sonicated for 4 minutes, topped with nitrogen and heated at 85°C for 1.5 hour. The sample was cooled at room temperature;0.5 mL chloroform extracted water was added and then 2 mL hexane, forming two layers. The upper organic layer was removed and flushed with nitrogen, capped, sealed with Teflon® tape and stored at -20°C until analysis.

The FAME was analyzed on a HP 6890 GC FID equipped with a 7683 autosampler. The GC column was a ZB wax+ (Phenomena, USA). The column length was 30 m with an internal diameter of 0.32 mm. The column temperature began at 65°C where it was held for 0.5 min. The temperature ramped to 195°C at a rate of 40°C/min, held for 15 min then ramped to a final temperature of 220°C at a rate of 2°C/min. This final temperature was held for 0.75 min. The carrier gas was hydrogen flowing at 2 mL/minute. The injector temperature started at 150°C and ramped to a final temperature of 250°C at a rate of 120°C/minute. The detector temperature stayed constant at 260°C. Peaks were identified using retention times from standards purchased from Supercool: 37 component FAME mix (Product number 47885-U), Bacterial acid methyl ester mix (product number 47080-U), PUFA 1 (Product number 47033) and PUFA 3 (product number 47085-U). Chromatograms were integrated using the Varian Galaxies Chromatography Data System, version 1.9.3.2.

2.3.2 *Amino acid analysis*

2.3.2.1 *Total amino acid (TAA)*

Larvae samples were ground in 2 mL Mille-Q water. Total amino acids were extracted and derivative using an EZ: faast™ Kit for Amino Acid Analysis of protein hydrolysates by GC-FID. From the ground sample, 0.5 mL was taken and added into a burned vial, to which 0.5 mL of HCl and phenol solution were added, and the sample was flushed with nitrogen, capped and kept in an oven at 110 °C for 24 hours. On returning to room temperature, 100 µL of sample hydrolysate was mixed with 200 µL of sodium carbonate solution to neutralize the solution and

the sample was mixed in a vortex until no bubbles were observed. Then 25 µL of the neutralized sample was taken, and 100 µL of an internal standard, norvaline (0.2 mM) and n-propanol, and 200 µL of water were added and passed through a sorbent tip. The sorbent material was then ejected in an eluting medium consisting of 3:2 sodium hydroxide/n-propanol. Fifty µL of chloroform and 100-µL iso-octane were added to the solution to form the organic layer containing the TAA, and 1 N hydrochloric acid was added to complete the derivatization, and the upper layer was taken. Samples ran on a Varian 3800 GC-FID to obtain peaks of FAA present, with the exception of taurine and arginine, which are not quantified with this kit. Peak areas were quantified comparing with a known quantity of internal standard.

2.3.2.2 *Free amino acid (FAA)*

Larvae samples were ground up in 2 mL Milli-Q water. Free amino acids were extracted and derivatized using an EZ: faast™ GC-FID Free (Physiological) Amino Acid Analysis Kit. A total of 100 µL of sample was mixed with 100 µL of an internal standard, norvaline (0.2 mM) and n-propanol, and passed through a sorbent tip. It was then washed with 200 µL of n-propanol and the sorbent material was then ejected in an eluting medium consisting of 3:2 sodium hydroxide/n-propanol. Fifty µL of chloroform and 100 µL iso-octane were added to the solution to form the organic layer containing the FAA, and 1 N hydrochloric acid was added to complete the derivatization. Samples ran on a Varian 3800 GC-FID to obtain peaks of FAA present, with the exception of taurine and arginine, which are not quantified with this kit. Peak areas were quantified comparing with a known quantity of internal standard.

2.4 *Statistical analysis*

Statistical analysis included analysis of variance (ANOVA) and Tukey's HSD multiple range comparison to test for significant differences among means of analyzed parameters. All analyses were done at the 95% confidence interval. ANOVA and post hoc analysis were done using Instat Software™.

3 Results

3.1 ***Lipid classes***

3.1.1 *Diets*

The lipid classes of diets are shown in Figure 1. The most important lipid classes in the diets were triacylglycerol (TG), sterol (ST), phospholipids (PL) and free fatty acids (FFA). The rotifers have PL as the highest lipid class, especially when enriched with protein hydrolysate. The Ori-Green enrichment increased the TG in the animals. However, both enrichments did not affect ST and FFA classes.

Artemia, enriched or not, presented the lowest values of PL when compared with rotifers and wild zooplankton, but presented the highest values of TG. The enrichments did not affect FFA, TG or PL classes, but increased significantly the ST class.

Wild zooplankton had almost the same amount of TG and FFA (≈ 25%), and PL and ST similar to the values of non-enriched rotifers.

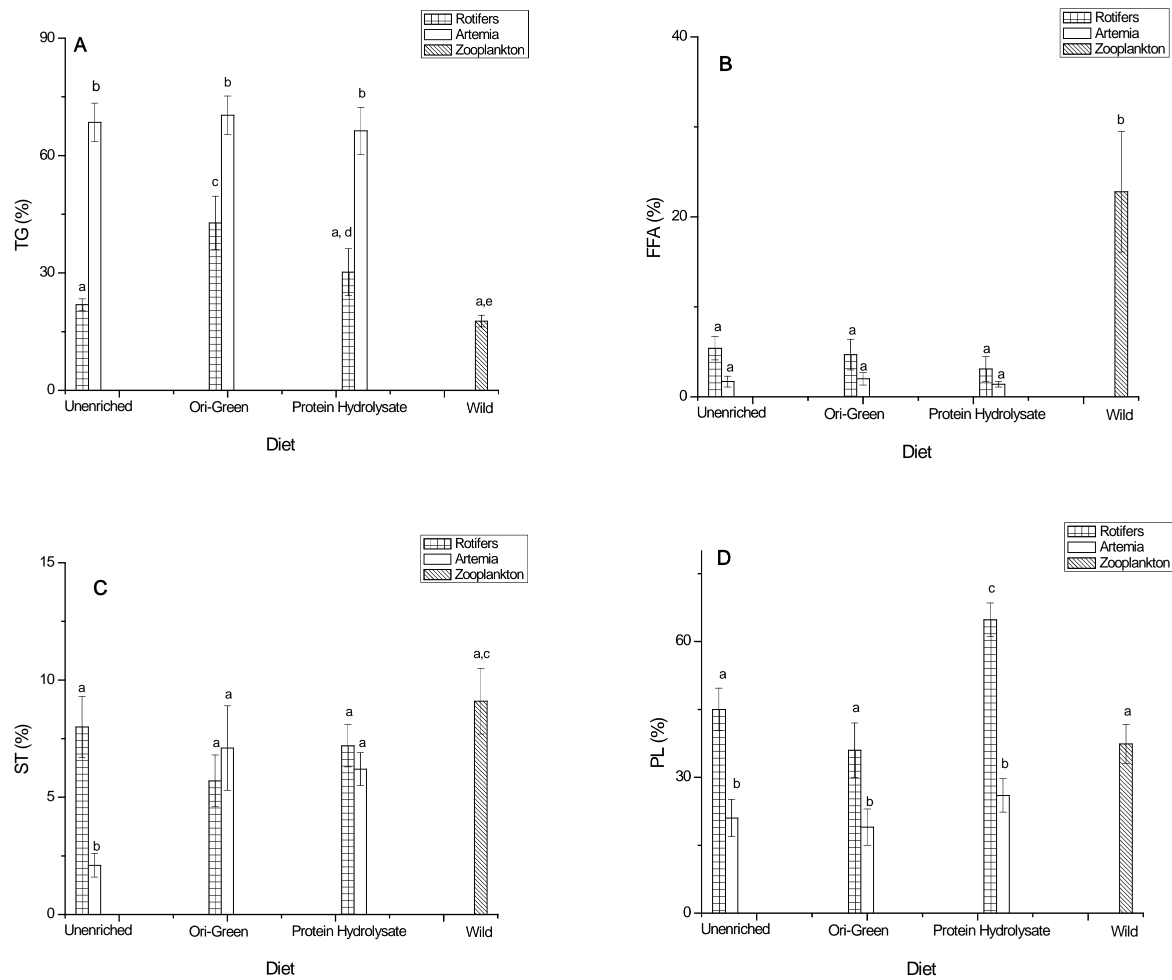

Figure 1: Total lipid classes in different diet treatments. A-Triacylglycerol (TG); B- Free fatty acids (FFA); C- Sterol (ST), D- Phospholipids (PL).

3.1.2 *Larvae*

The lipid classes of larvae are shown in Figure 2. The most important lipid class in the larvae was PL corresponding to ≈ 50% of total lipid classes. There was no significant difference in this lipid class between 9 and 11 mm, but there was a little reduction at 13 mm length, in all treatments, with the lowest value at 13 mm length in the RA-Zoo treatment. The highest values for ST was obtained at 9 mm length, and after this, there was a reduction in this class of lipid. On the other hand, TG increased with the length, and the highest values were obtained at RA-Zoo treatment at 13 mm length.

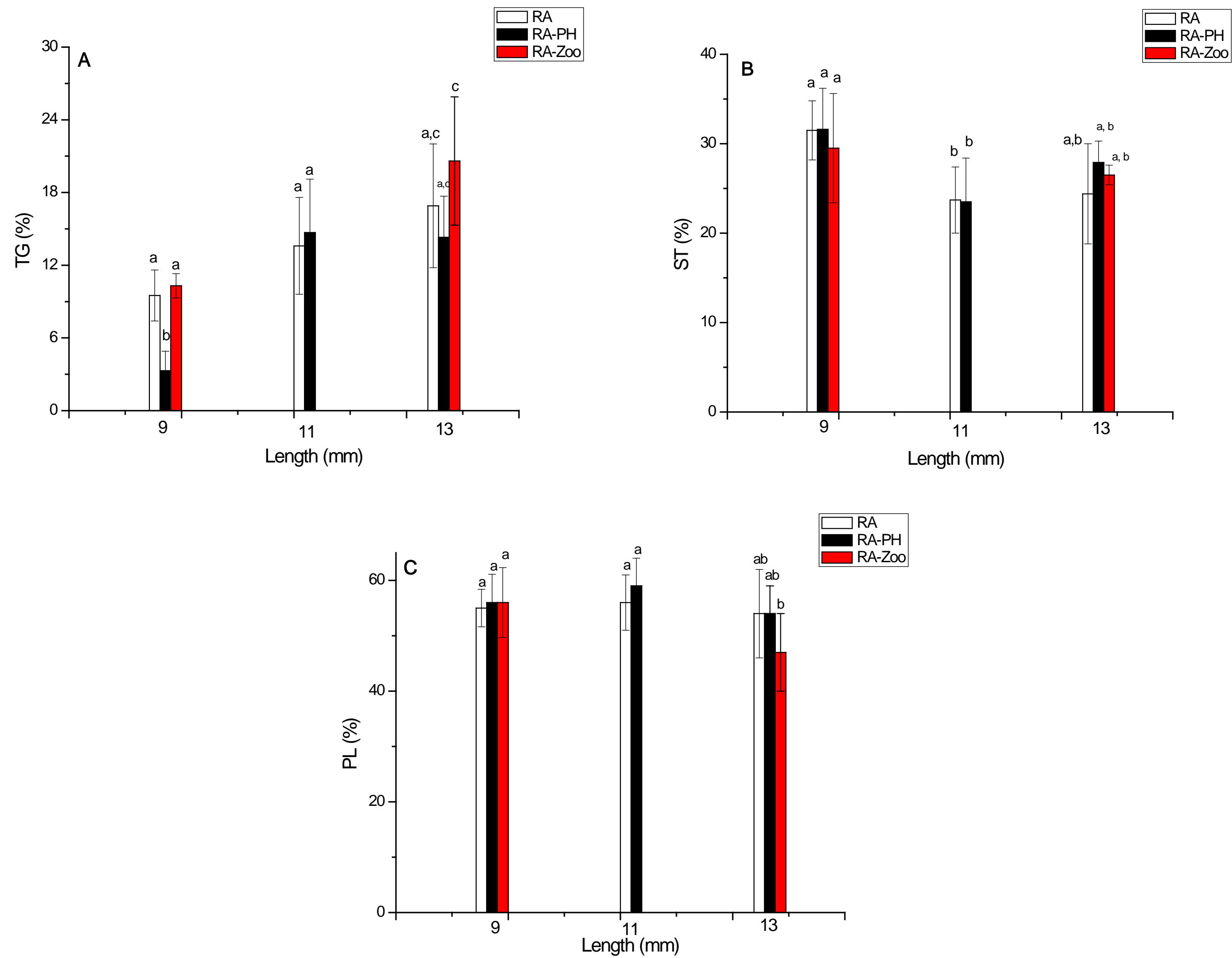

Figure 2: Total lipid classes in different larvae treatments (RA; RA-PH; RA-Zoo) and lengths (9, 11 and 13 mm of Atlantic cod (*Gadus morhua*).A- Triacylglycerol (TG); B- Sterol (ST), C - Phospholipids (PL).

3.2 ***FAMEs***

3.2.1 *Diets*

The enrichments, especially Ori-Green, increased DHA (22:6ω3) in rotifers while EPA (20:5ω3) was not so affected, and because of this the DHA/EPA ratio in the Ori-Green treatment was the highest (≈ 2.7) while the protein hydrolysate did not significantly affect this ratio, compared to the non enriched organisms (≈ 1.5).

The Ori-Green enrichment increased DHA and EPA in *Artemia*, and the DHA/EPA ratio was highest in *Artemia* in this treatment. The protein hydrolysate enrichment did not affect the DHA or EPA in *Artemia*, with similar results to the non-enriched organisms.

Wild zooplankton showed the highest values of DHA and EPA, and a DHA/EPA ratio close to 2. The sum of omega-3 fatty acids in zooplankton was responsible for almost 50% of the total fatty acids, close to that in *Artemia* and significantly higher than in rotifers (Fig 3).

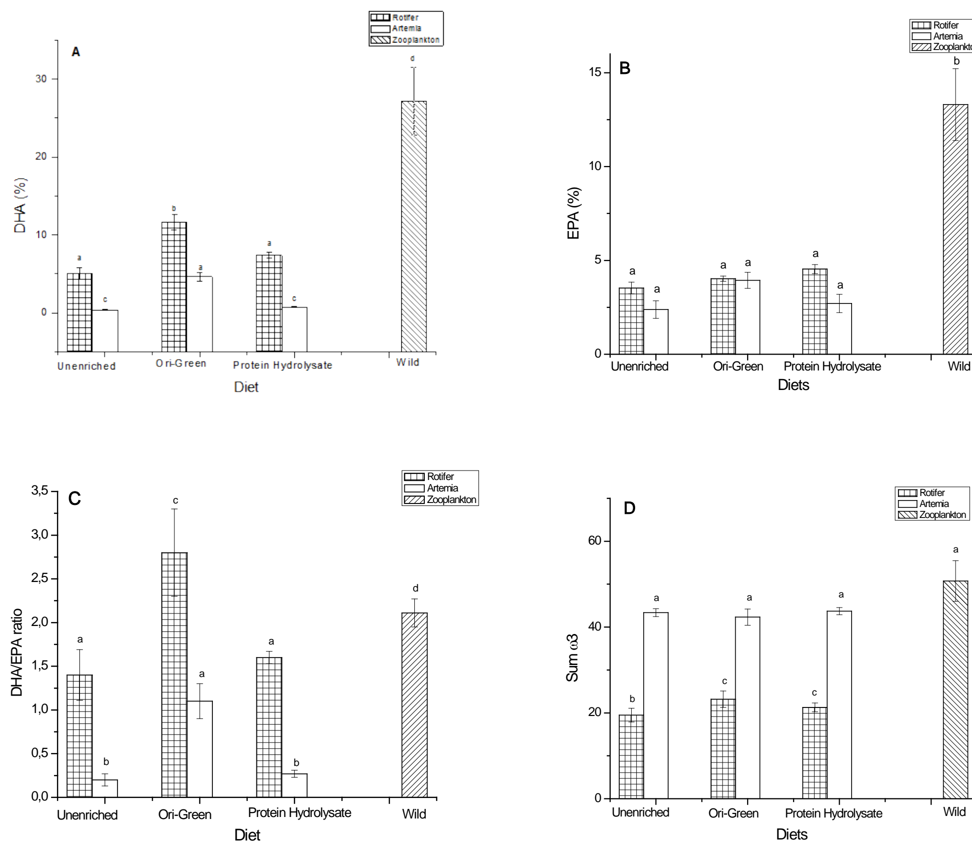

Figure 3: Fatty acid composition in different diet treatments. A-DHA; B- EPA; C-DHA/EPA ratio, D- Sum ω3.

3.2.2 *Larvae*

The larvae presented a reduction in the percentage of DHA according to length, while the EPA values increased with the length, so the highest DHA/EPA ratios were at 9 mm length – RA and RA-PH treatments. The highest values obtained for DHA, EPA and total percentage of omega-3 fatty acids occurred in RA-Zoo treatments (Fig 4).

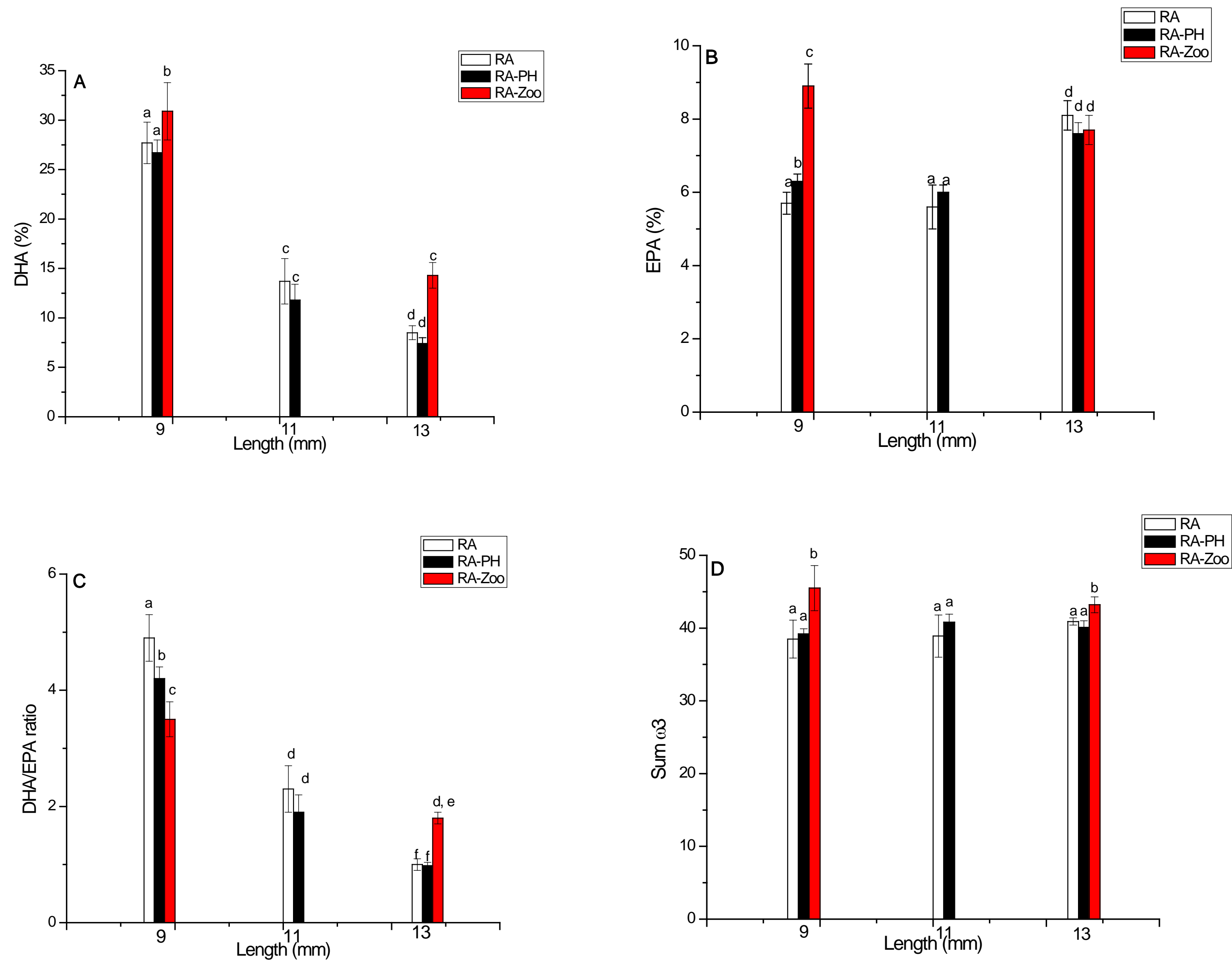

Figure 4: Fatty acid composition in different larvae treatments (RA; RA-PH. RA-Zoo) and lengths (9, 11 and 13 mm of Atlantic cod (*Gadus morhua*). A-DHA; B- EPA; C-DHA/EPA ratio, D- Sum ω3.

3.3 *Total AA*

3.3.1 *Diets*

The percentage of total protein, aromatic and essential amino acids obtained in the diets are shown in Figure 5.

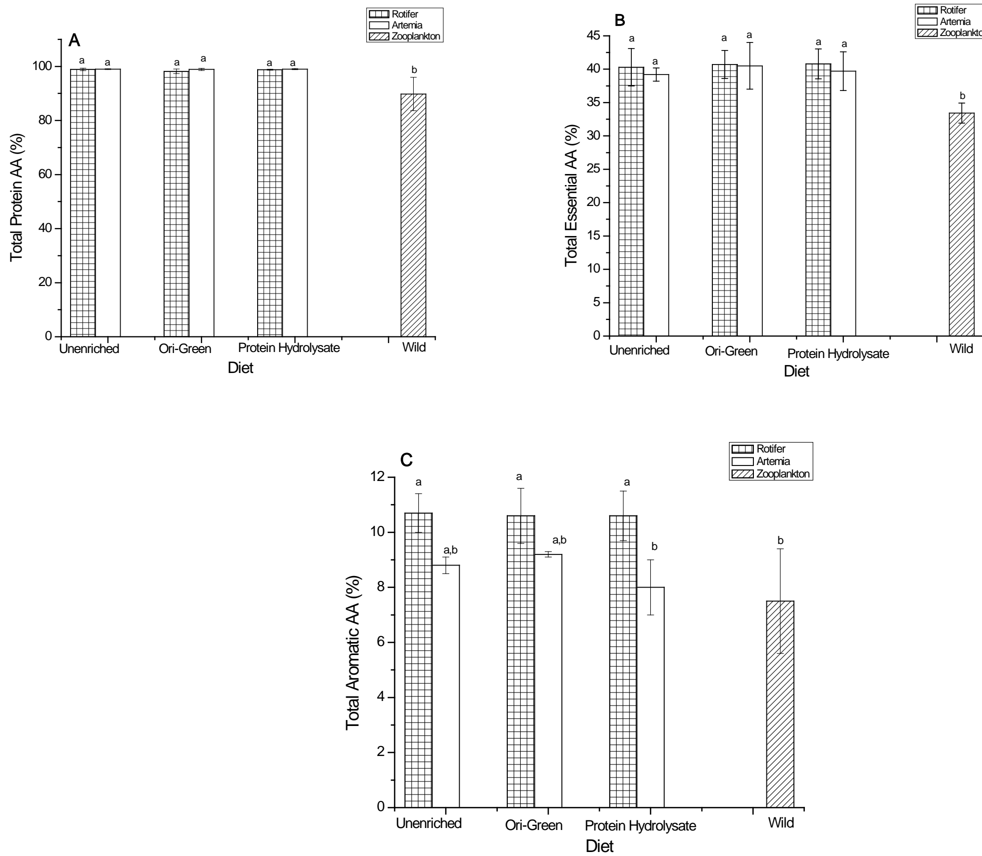

Figure 5: Total amino acids composition in different diet treatments. A-Protein; B- Essential; C-Aromatic.

3.3.2 *Larvae*

Total protein, aromatic and essential amino acids obtained in fish larvae at 9, 11 and 13 mm are shown in Figure 6.

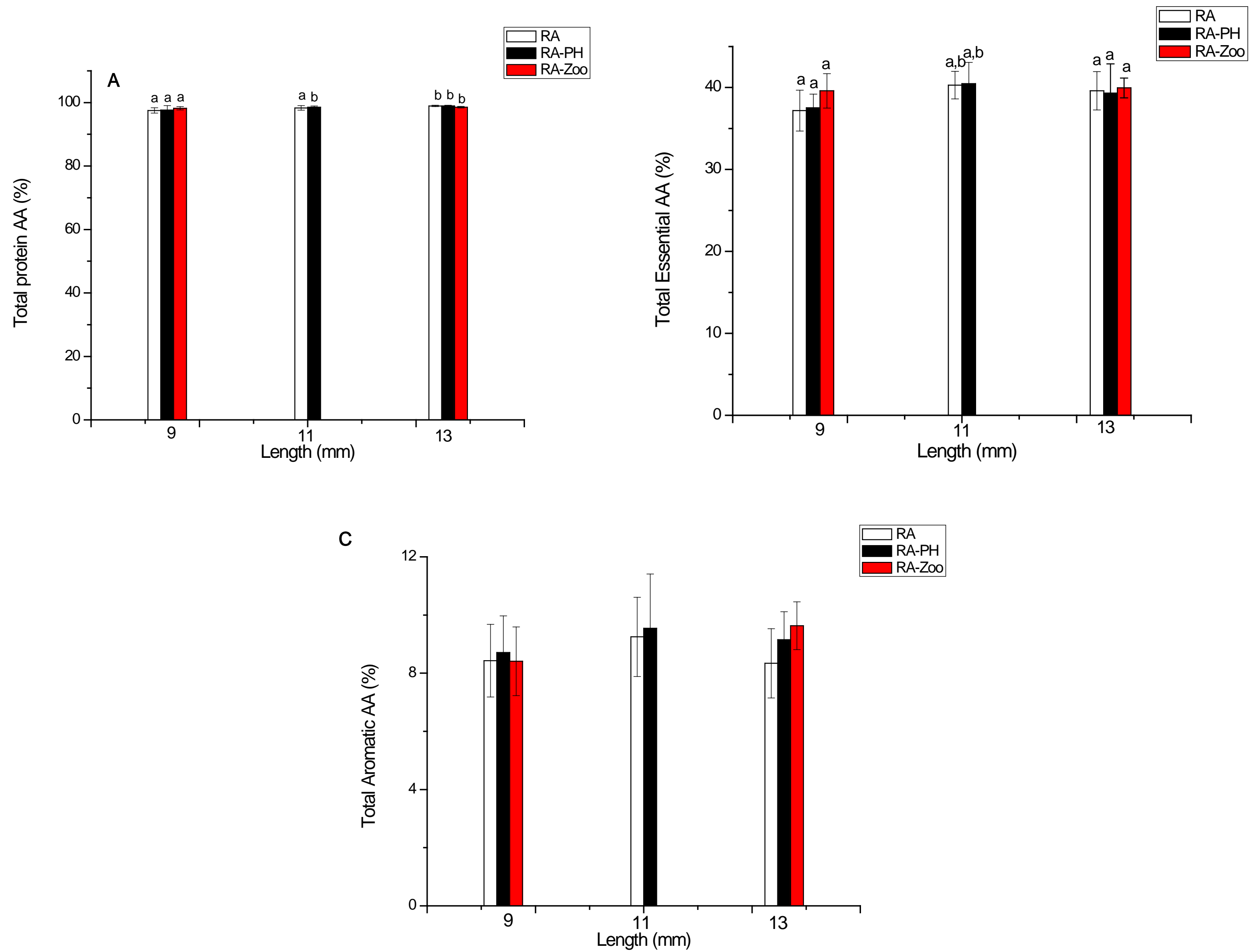

Figure 6: Total amino acids composition in different different larvae treatments (RA; RA-PH; RA-Zoo) and lengths (9, 11 and 13 mm of Atlantic cod (*Gadus morhua*).A-Protein; B- Essential; C-Aromatic.

3.4 ***Free AA***

Larvae

Free protein, aromatic and essential amino acids obtained in fish larvae at 9, 11 and 13 mm are shown in Figure 7.

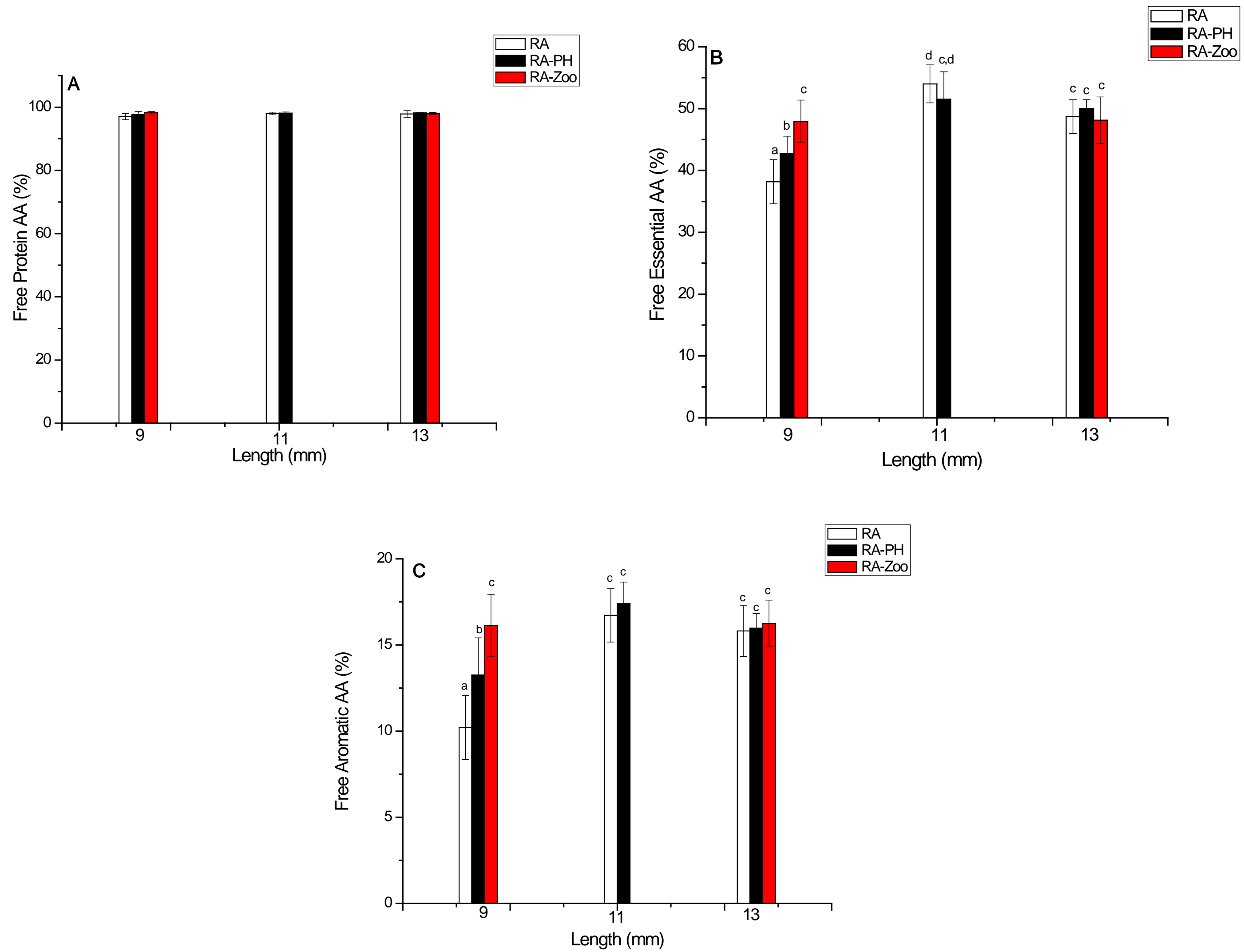

Figure 7: Free amino acids composition in different larvae treatments (RA; RA-PH; RA-Zoo) and lengths (9, 11 and 13 mm of Atlantic cod (*Gadus morhua*).A-Protein; B- Essential; C-Aromatic.

4 Discussion

The first feeding of marine fish larvae with high nutritional quality is important because food quality and quantity affect health and regular vertebral formation of fish (Hamre, 2006; Kainz et al., 2004; Oie and Olsen, 1997; Payne et al., 1998). Lipids and amino acids are some essential nutrients that affect fish development.

Triacylglycerols can be used for short-term energy needs while PL are important as storage lipids in zooplankton and as a structural component of cell membranes (Conelly et al., 2012; Jeffs et al., 2001; Lee et al., 2006). These two lipid classes are the most affected in the enriched treatments tested in this study, and this can alter the nutritional values of the organisms. The unenriched rotifers present PL and FFA percentages close to that obtained by Garcia et al. (2008a), but PL was lower than obtained by Park et al (2006). TG percentage is lower than obtained by Garcia et al. (2008a) and close to that obtained by Park et al. (2006), but ST is higher in the present work when compared with the results obtained by Garcia et al. (2008a) and Park et al. (2006). These differences can also be observed in the enriched rotifers treatments, while Garcia et al. (2008a) observed an increase in TG and a decrease in PL in the enriched animals, we observed an increase in TG in both enrichments tested and in PL in the protein hydrolysate enrichment. Unenriched *Artemia* presented a higher PL percentage in this work than found by Garcia et al. (2008b). The *Artemia* enrichments done by these authors resulted in an increase in the percentage of PL and a decrease in TG. In this work the enrichments did not affect PL or TG, but increased ST when compared to unenriched organisms.

Copepod is good for first feeding small marine fish larvae and can be used as a complement to rotifers and *Artemia* as live feed (Olivotto et al., 2008), providing higher pigmentation, growth and survival, and lower incidence of diseases (Støttrup, 2000). Better results were obtained when combining copepods and *Artemia* in turbot feeding, improving survival when compared to rotifers and *Artemia* (Støttrup and Norsker, 1997).

The polyunsaturated fatty acids (PUFA) are a component of membrane PL, important for normal visual and neural development in larvae (Arts et al., 2001; Estevez et al., 1998), and high amounts of PUFA can be obtained in copepods (Pond and Tarling, 2011), but apparently *Artemia* is nutritionally deficient for cold water fish larvae, that have high ω3 HUFA requirements (Nanton and Castel, 1998; Olsen et al., 1997).

The wild zooplankton had DHA/EPA ratios close to 2, considered ideal for larval development (Bell et al., 2003; Støtrupp et al., 1999). Food with low DHA and EPA results in lower growth and survival rates, as well as incomplete pigmentation in larvae of fish (Olsen et al., 1997, Watanabe, 1993). The enrichments of rotifers and *Artemia* can increase these fatty acids and this ratio, resulting in a better live food (Evjemo and Olsen, 1997; Evjemo et al., 1997; Rainuzzo et al., 1994). With the results observed in the enrichments tested, we obtained the best results in the Ori-Green treatments of rotifers and *Artemia*. The results obtained here were smaller than obtained for Evjemo and Olsen (1997) using DHA Selco and Super Selco enrichments (2.2). Even enriched, *Artemia* is a poorer option in relation to copepods and rotifers (Bell et al 2003). The values of DHA, EPA and the DHA/EPA ratios in rotifers were higher in this study in unenriched rotifers than obtained by Garcia et al. (2008a) and by Park et al. (2006), but with the enrichments, these authors found higher values of those fatty acids. In the present study, Ori-Green enrichment increased DHA and the DHA/EPA ratio, but with lower values than obtained by Garcia et al. (2008a). The values obtained for unenriched *Artemia* were similar to those obtained by Garcia et al (2008b), and the Ori-Green enriched animals have results close to those obtained with AquaGrow enrichment.

Cod (*Gadus morhua*)has great potential for aquaculture and is easy to cultivate. The larvae need long chain HUFAs, and more lipids in diets apparently promote better growth and survival, providing more energy to capture more live prey (Brown et al., 2003, O'Brien-MacDonald et al., 2006). The ability to digest and absorb ω-3 HUFA from PL or TG can promote fish growth and avoid fatty acid deficiency in marine fish larvae (Izquierdo et al., 2000).

Koedjik et al. (2010) obtained higher survival in cod larvae fed rotifers during 36 days, but survival between 36 and 50 days was better in larvae fed zooplankton. Larvae that changed diet from rotifers to zooplankton at 22 days increased in mass faster than larvae that were continually fed rotifers, but lower mass when larvae were fed zooplankton and changed to rotifers when compared with animals that were continually fed zooplankton. The authors concluded that the first feeding diet in larval and juvenile cod affected growth, and that rotifers are not recommended for larval nutrition after 20 days, because cod need a higher variety of nutrients after 22 days. Cod larvae have a high growth rate potential (Hamre, 2006), and in the present study we observed a faster growth in cod fed with zooplankton complementing rotifers and *Artemia*, with the larvae reaching 13 mm length before the treatments that were fed just with

rotifers and *Artemia.* This agrees with Busch et al. (2010) who state that rotifers are not enough to achieve the growth potential of cod. Imsland et al. (2006) observed that providing rotifers as food during the larval stage is insufficient in quality for juvenile fish, and the larvae fed zooplankton had higher growth rates, food intake, feed conversion rates and less skeletal deformities than rotifer groups.

Comparing the lipid composition of the larvae in this study (35 dph) with that observed by Garcia et al. (2008a), with larvae at 37 dph, we obtained higher values of TG (14 – 20%) in comparison with the other authors (1.3 - 2.1%), almost the same amount of ST (25 – 27.5% here and 24 – 33% in the other study) and smaller values of PL (45 – 57%) than the other authors (61 – 70%). The difference between these studies is in the initial feeding, while in the present study the larvae received rotifers, *Artemia* and wild zooplankton until 35 dph, in different combinations and according to age, in the study of Garcia et al (2008a), the larvae received just rotifers as live feed until 37 dph. This different initial live feed can be the responsible for the differences observed.

Park et al. (2006) suggest that larval cod require a high DHA/EPA ratio, and DHA levels and DHA/EPA ratios of rotifers influenced the growth and survival. Olsen et al. (1999) found DHA in halibut larvae decreased from day 0 (44.3%) to day 13 (19.3%), as well as DHA/EPA ratio, and the same was observed in this study with cod larvae. Comparing with the results of Garcia et al. (2008a), the values obtained in this study for DHA and DHA/EPA ratio were smaller, while we had a DHA around 12.5%, decreasing from 9 (~35%) to 13 mm length, and a DHA/EPA ratio around 2.1, decreasing from 9 to 13 mm too, the authors obtained DHA from 26 to 36% in different treatments, and DHA/EPA ratios varying from 8.6 to 12.1. Our EPA values were higher (5.5 – 6%) than in the other study (2.3 – 3.4%) and we believe that this difference is because of the food supplied to the larvae – different combinations in this study and just rotifers in the study of Garcia et al. (2008a).

Free amino acids are important in the osmoregulation, metabolism and nutritional requirements of larvae, providing energy for metabolic reactions (Finn and Fyhn, 2010; Zhu et al., 2003). Dietary AA are important to achieve the best growth and homeostasis results, providing carbon skeletons to carbohydrate and lipid synthesis in fish (Bakke et al., 2010; Evans et al., 2000, Zhu et al., 1997) and, apparently, live prey (rotifers and *Artemia*) does not affect the

larvae AA profile, being not adequate to provide the amino acids requirements for larval fish (Conceição et al., 2003).

In present study we observed that in relation to the percentage of total protein and essential amino acids, no difference was detected between rotifers and *Artemia*, but wild zooplankton had the lowest values in these percentages. Rotifers had the highest values of total aromatic amino acids, significantly higher than *Artemia* and wild zooplankton.

In relation to the total protein amino acids, a small increase was observed in the percentage of these amino acids with increasing length, especially at 13 mm. The same was observed in relation to the essential amino acids, but the highest values were obtained at 11 mm. The aromatic amino acids did not present significant changes with the length.

The free protein amino acids did not change with the length or according to the treatments in the larvae. On the other hand, the essential and aromatic amino acids had changes according to the length, and treatment, only at 9 mm length, where the differences were higher, but at 11 or 13 mm, there were no more differences between treatments.

5 Conclusions

Based on our results we can conclude that wild zooplankton constitutes the best option to feed Atlantic cod, even when used as a complement to rotifers and *Artemia.*

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5. CONCLUSÕES GERAIS

a) A composição bioquímica da clorofícea *Ankistrodesmus gracilis* variou em função de diferentes concentrações de fósforo e de cobre no meio, sendo que a produção de carboidratos foi mais afetada do que a de proteínas, enquanto as classes lipídicas triglicerídeos e fosfolipídios sofreram as maiores alterações de acordo com a disponibilidade de nutrientes.

b) A clorofícea *Ankistrodesmus gracilis* apresentou uma maior sensibilidade ao cobre em situações com menos fósforo disponível no meio.

c) Os organismos do zooplâncton avaliados apresentam boas quantidades de carboidrato e proteína, especialmente adultos de branconeta. O perfil de lipídios de branconeta se altera de acordo com o sexo e idade, o que pode refletir em alterações de ácidos graxos.

d) Houve influência dos diferentes organismos alimentares (naturais e enriquecidos) na composição lipídica e de aminoácidos do bacalhau do Atlântico (*Gadus morhua*), sendo que o zooplâncton natural é recomendado como um complemento aos rotíferos e *Artemia*.

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