Biochemical Composition of Protists: Dependency on Diet and Trophic Mode and Consequences for their Nutritional Quality

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ABBREVIATIONS

P	phosphorus
N	nitrogen
C	carbon
SAFA	saturated fatty acids
MUFA	monounsaturated fatty acids
PUFA	polyunsaturated fatty acids (>1 double bond)
HUFA	highly unsaturated fatty acids (≥4 double bonds)
EPA	eicosapentaenoic acid
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
SDA	stepwise discriminant analysis
P	significance level
ILL	incipient limiting level
rpm	rotations per minute
DAPI	4,6-diamidino-2-phenylindol
BSA	bovine serum albumin
MVA	mevalonate pathway of sterol synthesis
MEP	methylerythritol-phosphate pathway of sterol synthesis
CoA	coenzyme A

Organisms are chemical entities
and are produced, maintained,
and propagated by chemical reactions,
albeit in the form of highly complex
coupled networks, which are the
product of evolution.

Sterner & Elser 2002

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SUMMARY

Variation in the rate at which organic carbon is transferred across trophic levels in aquatic food webs is quite large, which may be partially related to differences in the food quality of planktonic prey organisms. Organic carbon is allocated into various biochemical molecules that differ greatly in their energy content and essentiality for zooplankton predators. Thus, the measurement of the biochemical composition of planktonic organisms is an efficient tool to evaluate their nutritional quality as prey. Among the most important compounds conferring nutritional quality on planktonic prey organisms are essential fatty acids, amino acids, and more recently, sterols. Although it has been widely accepted that heterotrophic protists are an important component linking the microbial and the classical food webs, relative little is known about their biochemical composition and resulting nutritional quality as prey. In this thesis I have considered two main aspects of the biochemical composition of protists: (1) whether the biochemical composition depends on the dietary resources or the trophic mode of the protist; and (2) whether biochemical composition determines nutritional quality of protists as prey for zooplankton.

The fatty acid, amino acid, and sterol composition of four heterotrophic protist species was analysed and compared to the composition of the respective dietary resources. The algivorous ciliates *Balanion planctonicum* and *Urotricha farcta* were fed the cryptomonad *Cryptomonas phaseolus*; the bacterivorous ciliate *Cyclidium* sp. and the flagellate *Chilomonas paramecium* were fed bacteria grown on rice corns. The fatty acid and amino acid composition of the heterotrophic protists generally resembled the composition found in their diet. However, the protists accumulated fatty acids and amino acids. *B. planctonicum* and *U. farcta* showed higher carbon-specific concentrations of monounsaturated and some polyunsaturated fatty acids than their algal diet *C. phaseolus*. Moreover, except for tryptophan, valine, and lysine, higher carbon-specific amino acid concentrations were observed in both *B. planctonicum* and *U. farcta* than in *C. phaseolus*. *Cyclidium* sp. and *C. paramecium* had higher carbon-specific concentrations of polyunsaturated fatty acids and amino acids than their diet, except for histidine, methionine, and leucine. Cell-specific fatty acid concentrations were generally higher in algivores than in bacterivores, while cellular-specific amino acid concentrations were similar among protists. The sterol composition of the protists was less dependent on dietary composition than the fatty acid and amino acid composition. Ergosterol was the main sterol in the algal diet *C. phaseolus*, whereas stigmasterol was dominant in its predators *B.*

planctonicum and *U. farcta*. The bacterial diet was rich in cholesterol and sitosterol, whereas cholesterol and stigmasterol were the major sterols in bacterivores *Cyclidium sp.* and *C. paramecium*. Higher sterol concentrations in the protists than in their diet indicate sterol accumulation by the protists. Efficient ingestion and assimilation of lipids and amino acids, preferential metabolism of carbohydrated compounds, and synthesis of some biochemicals are mechanisms likely underlying accumulation of biochemical compounds in the heterotrophic protists.

To evaluate whether the biochemical composition depends on the trophic mode of protist, the fatty acid and sterol composition of autotrophically, mixotrophically, and heterotrophically cultured flagellates of a species from the genus *Ochromonas* were evaluated. The trophic mode strongly affected the biochemical composition of *Ochromonas* sp.. Especially the concentrations of polyunsaturated fatty acids decreased from autotrophy, via mixotrophy to heterotrophy. Discriminant analyses identified polyunsaturated fatty acids as the biochemical components which varied the most among *Ochromonas* sp. of different trophic modes. As several protist species exhibit different trophic modes within the same species (e.g. *Ochromonas* sp. and other chrysophyceae) – a nutritional flexibility that enables them to subsist under different environmental conditions – a great variability in their nutritional quality is expected for their predators.

The role of the biochemical composition in determining the nutritional quality of heterotrophic protists as prey was tested in population growth and reproduction experiments, using the rotifer *Keratella quadrata* as model predator. Several polyunsaturated fatty acids, three sterols (desmosterol, ergosterol, stigmastanol), and the amino acid leucine in the heterotrophic protists were significantly correlated with the rotifer's egg production. However, no correlation was observed between protists' biochemistry and population growth rates of *K. quadrata*. Based on the correlative evidences, the effects of single fatty acids on *Keratella*'s performance were tested by artificially supplementing *Chilomonas paramecium* with the fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The nutritional quality of *C. paramecium* was significantly enhanced by supplementing the flagellate with DHA. EPA effects were weaker and not significant. However, effects were again only found for the egg production but not for population growth of *K. quadrata*.

In the present thesis, I provided evidence that the biochemical composition of protists depends on their dietary resources as well as on their trophic mode.

However, heterotrophic protists exhibited the potential to accumulate or even modify biochemical substances obtained from their diet. Moreover, the biochemical composition of the studied protists influenced the reproduction of a zooplankton predator. My results thus highlight the necessity to incorporate heterotrophic protists into food quality studies, because of their ability to modify the biochemical composition of organic matter at an early stage in aquatic food webs, i.e. at the interface between algae/bacteria and the mesozooplankton. Considering the high energy density and essentiality of some biochemical compounds (such as lipids), small biochemical modifications at this early stage may have profound consequences for matter and energy transfer through the entire food web.

ZUSAMMENFASSUNG (GERMAN)

Die Variation in den Transferraten mit denen organischer Kohlenstoff in aquatischen Nahrungsnetzen auf die nächste trophische Ebenen übertragen wird, ist groß. Dies könnte teilweise auf Unterschiede in der Nahrungsqualität planktischer Beuteorganismen zurückzuführen sein. Organischer Kohlenstoff liegt in den Beuterganismen in Form verschiedener biochemischen Stoffe vor, die sich bezüglich ihres Energiegehaltes und ihrer Essentialität für planktische Räuber unterscheiden. Die Messung der biochemischen Zusammensetzung planktischer Beuteorganismen stellt somit die geeignete Methode zur Bestimmung ihrer Nahrungsqualität dar. Essentielle Fettsäuren, Aminosäuren und seit neuestem auch Sterole sind die am besten untersuchten Kohlenstoffverbindungen, die die Nahrungsqualität planktischer Beuteorganismen bestimmen. Trotz der allgemein akzeptierten Schlüsselstellung heterotropher Protisten als Bindeglied zwischen dem mikrobiellen und dem klassischen Nahrungsnetz ist noch wenig über ihre biochemische Zusammensetzung und ihre daraus resultierende Nahrungsqualität bekannt. In der vorliegenden Doktorarbeit habe ich drei Hauptaspekte der Biochemie von Protisten untersucht, nämlich ob die biochemische Zusammensetzung von Protisten (1) von deren Nahrungsgrundlage und Ernährungsweise abhängt und (2) ihre Nahrungsqualität für räuberisches Zooplankton bedingt.

Hierzu wurde die Fettsäure-, Aminosäure- und Sterolzusammensetzung von vier heterotrophen Protistenarten untersucht und mit der biochemischen Zusammensetzung ihrer Nahrungsgrundlage verglichen. Die algivoren Ciliaten *Balanion planctonicum* und *Urotricha farcta* wurden auf der Cryptomonade *Cryptomonas phaseolus* kultiviert, der bakterivore Ciliat *Cyclidium* sp. und der bakterivore Flagellat *Chilomonas paramecium* auf Bakterienkulturen, die auf Reiskörnern wuchsen. Die Fettsäure- und Aminosäurezusammensetzung der heterotrophen Protisten ähnelte generell der ihrer Nahrung. Es trat jedoch eine Akkumulation von Fettsäuren und Aminosäuren in den Protisten auf. *B. planctonicum* and *U. farcta* wiesen höhere kohlenstoffspezifische Konzentrationen von allen einfach ungesättigten und einigen mehrfach ungesättigten Fettsäuren auf als ihre Futteralge *Cryptomonas*. Weiterhin wurden in *B. planctonicum* and *U. farcta*, mit Ausnahme von Tryptophan, Valin und Lysine, höhere kohlenstoffspezifische Aminosäurekonzentrationen gemessen als in *Cryptomonas. Cyclidium* sp. und *C. paramecium* wiesen zudem, mit Ausnahme von Histidin, Methionin und Leucin, höhere kohlenstoffspezifische Konzentrationen von mehrfach ungesättigten Fettsäuren und Aminosäuren auf als

ihr Futter. Bei den algivoren Protisten traten generell höhere zellspezifische Fettsäurekonzentrationen auf als bei den bakterivoren. Die zellspezifischen Aminosäurekonzentrationen der Protisten wiesen keine Unterschiede auf. Die Sterolzusammensetzung der Protisten hing weniger stark von der der Nahrung ab als die Fettsäure- und Aminosäurezusammensetzung. Ergosterol war das wichtigste Sterol der Alge *Cryptomonas*, wohingegen Stigmasterol das wichtigste Sterol ihrer Räubern *B. planctonicum* und *U. farcta* war. Die Bakterienkultur war reich an Cholesterol and Sitosterol, wohingegen bei den bakterivoren Protisten *Cyclidium sp.* and *C. paramecium* Cholesterol und Stigmasterol dominierte. Wiederum traten in den Protisten höhere Sterolkonzentrationen als in ihrer Nahrung auf. Eine solche Akkumulation von biochemischen Substanzen kann auf effiziente Ingestion und Assimilation, bevorzugte Umsetzung von Kohlenhydraten oder Synthese zurückzuführen sein.

Weiterhin wurde die Fettsäure- und Sterolzusammensetzung von autotroph, mixotroph und heterotroph kultivierten Flagellaten einer Art der Gattung *Ochromonas* untersucht, um zu prüfen, ob die biochemische Zusammensetzung von Protisten von ihrer Ernährungsweise abhängt. In der Tat bedingte die Ernährungsweise des Flagellaten *Ochromonas* sp. stark dessen biochemische Zusammensetzung. Insbesondere die Konzentrationen an mehrfach ungesättigten Fettsäuren waren in autotrophen Flagellaten höher als in mixotrophen und in mixotrophen höher als in heterotrophen. Mittels Diskriminanzanalysen konnte zudem gezeigt werden, daß mehrfach ungesättigten Fettsäuren die biochemischen Substanzen waren, die am stärksten zwischen *Ochromonas* sp. unterschiedlicher Ernährungsweise variierten. Da einige Protistenarten mit verschiedenen Ernährungsweisen innerhalb derselben Art vorkommen (z.B. *Ochromonas* spp. und andere Chrysophyceen) – diese Flexibilität ermöglicht es ihnen bei unterschiedlichen Umweltbedingungen zu existieren – ist zu vermuten, daß solche Arten große Unterschiede in ihrer Nahrungsqualität für Räuber aufweisen.

Die Frage, ob die biochemische Zusammensetzung heterotropher Protisten ihre Nahrungsqualität für planktische Räuber bedingt, wurde anhand des Populationswachstums und der Reproduktion der Rotatorie *Keratella quadrata*, einem wichtigen planktischen Räuber, untersucht. Verschiedene mehrfach ungesättigte Fettsäuren, drei Sterole (Desmosterol, Ergosterol, Stigmastanol) und die Aminosäure Leucin in den Protisten waren signifikant mit der Eiproduktion von *K. quadrata* korreliert. Es wurden jedoch keine Korrelationen zwischen der Biochemie der Protisten und dem Populationswachstum von *K. quadrata* gefunden. Zur näheren Untersuchung dieser korrelativen Befunde wurde der

Flagellat *Chilomonas paramecium*, der eine geringe Nahrungsqualität aufwies, künstlich mit den mehrfach ungesättigten Fettsäuren Eicosapentaensäure (EPA) und Docosahexaensäure (DHA) supplementiert und an *K. quadrata* verfüttert. DHA erhöhte die Nahrungsqualität von *C. paramecium* signifikant. Die Wirkung von EPA war schwächer und nicht signifikant. Diese Effekte wirkten wiederum nur auf die Eiproduktion, nicht aber auf das Populationswachstum von *K. quadrata*.

In der vorliegenden Doktorarbeit konnte gezeigt werden, daß die biochemische Zusammensetzung von Protisten von deren Nahrungsgrundlage und Ernährungsweise abhängt. Heterotrophe Protisten zeigten jedoch auch das Potential von ihrer Nahrung erhalten biochemische Verbindungen zu akkumulieren oder sogar zu verändern. Weiterhin bestimmte die biochemische Zusammensetzung der Protisten die Reproduktionsleistung eines planktischen Räubers. Die Ergebnisse meiner Untersuchungen verdeutlichen somit die Notwendigkeit, heterotrophe Protisten in Studien zur Nahrungsqualität zu berücksichtigen, da sie das Potential besitzen, die biochemische Zusammensetzung organischer Materie schon auf einer unteren Ebene des aquatischen Nahrungsnetzes zu verändern, nämlich beim Übergang zwischen Algen/Bakterien und dem Mesozooplankton. Aufgrund der hohen Energiedichte und Essentialität verschiedener biochemischer Substanzen (wie z.B. Lipiden), können kleine Änderungen in der Biochemie der organischen Materie auf einer so niedrigen trophischen Ebene bereits weitreichende Folgen für den Stoff- und Energiefluß durch das gesamte Nahrungsnetz nach sich ziehen.

RESUMO (PORTUGUESE)

A considerável variação nas taxas de transferência de biomassa carbônica ao longo de níveis tróficos em cadeias alimentares aquáticas provavelmente se deve a diferenças na qualidade alimentar dos organimos planctônicos. O carbono na biomassa se encontra alocado na forma de diferentes compostos bioquímicos, alguns destes considerados essenciais para muitos predadores zooplanctônicos. Desta forma, a composição bioquímica é uma ferramenta eficiente para se avaliar a qualidade nutricional dos organismos planctônicos como presas para o zooplâncton. Dentre os compostos bioquímicos mais bem relatados como determinantes da qualidade nutricional de presas planctônicas, encontram-se os ácidos graxos, aminoácidos e, mais recentemente, esteróis. Embora a importância de protistas heterotróficos como um elo de ligação entre as cadeias microbiana e clássica seja bem reconhecida, pouco se sabe sobre a composição bioquímica e conseqüente qualidade nutricional de protistas heterotróficos. Neste trabalho foram considerados dois aspectos fundamentais da composição bioquímica de protistas: (1) se a mesma depende do recurso alimentar ou do modo trófico do protista e (2) se a composição bioquímica de protistas determina sua qualidade nutricional para predadores zooplanctônicos.

A composição de ácidos graxos, aminoácidos e esteróis de quatro espécies de protistas heterotróficos foi analisada e comparada com a composição de suas respectivas dietas. Os ciliados algívoros *Balanion planctonicum* e *Urotricha farcta* foram alimentados com *Cryptomonas phaseolus*; o ciliado bacterívoro *Cyclidium* sp. e o flagelado *Chilomonas paramecium* foram mantidos com bactérias cultivadas em grãos de arroz. A composição de ácidos graxos e aminoácidos dos protistas geralmente refletiu a composição da dieta, mas acumulação de compostos bioquímicos foi observada na maioria dos casos. *B. planctonicum* e *U. farcta* apresentaram concentrações (por biomassa de carbono) mais elevadas de ácidos graxos monoinsaturados e alguns poliinsaturados do que a dieta algal. Ainda, exceto por triptofano, valina e lisina, concentrações mais elevadas de aminoácidos foram observados em ambos *B. planctonicum* e *U. farcta* do que em *Cryptomonas*. *Cyclidium* sp. e *C. paramecium* apresentaram concentrações mais elevadas de ácidos graxos poliinsaturados e de aminoácidos do que a dieta bacteriana, exceto para histidina, metionina e leucina. Protistas algívoros apresentaram concentrações celulares mais elevadas de ácidos graxos do que bacterívoros, enquanto nenhum padrão foi observado para os aminoácidos. A composição de esteróis, por sua vez, foi mais independente do recurso alimentar. Por exemplo, ergosterol foi o principal eserol em *C. phaseolus*, enquanto

estigmasterol predominou em *B. planctonicum* e *U. farcta*. A dieta bacteriana foi rica em colesterol e sitosterol, enquanto *Cyclidium sp.* e *C. paramecium* apresentaram respectivamente colesterol e estigmasterol como principais esteróis. Concentrações mais elevadas de esteróis nos protistas do que em suas dietas sugere novamente a acumulação bioquímica nos protistas. Características metabólicas tais como eficiência de ingestão e assimilação, metabolismo preferencial de carboidratos e biosíntese são prováveis mecanismos explicando a acumulação de compostos bioquímicos nos protistas heterotróficos.

O modo trófico – autotrofia, mixotrofia, heterotrofia – afetou consideravelmente a composição bioquímica dos protistas. Foram analisadas as composições de ácidos graxos e esteróis do flagelado *Ochromonas* sp. crescendo como autótrofo, mixotrófico e heterotrófico. Em especial as concentrações de ácidos graxos poliinsaturados apresentaram uma pronunciada redução à medida em que o modo trófico passou da autotrofia, via mixotrofia, para a heterotrofia. Segundo as análises discriminantes, ácidos graxos poliinsaturados foram o parâmetro bioquímico variando mais intensamente entre *Ochromonas* sp. de diferentes modos tróficos, assim como entre diferentes espécies de protistas heterotróficos. O fato de que uma única espécie protista (como *Ochromonas* sp. e outras chrysophyceae) é capaz de exercer diferentes modos tróficos – uma flexibilidade nutricional que confere à essa espécie a capacidade de viver em diferentes ambientes – uma considerável variação na composição bioquímica, e por conseguinte, na qualidade nutricional pode ser esperada por um predador desta espécie.

O papel da composição bioquímica em determinar a qualidade nutricional de protistas heterotróficos foi testada durante experimentos de crescimento populacional e reprodução, usado o rotífero *Keratella quadrata* como predador-modelo. O conteúdo de vários ácidos graxos poliinsaturados, três esteróis (desmosterol, ergosterol, and stigmastanol) e do aminoácido leucina nos protistas heterotróficos correlacionou-se significantemente com a produção de ovos do rotífero. No entanto, nenhuma correlação foi observada com as taxas de crescimento populacional de *K.quadrata*. Com base nas evidências correlativas encontradas, os efeitos limitantes de ácidos graxos (EPA e DHA) sobre a performance de *Keratella* foram testados por meio de suplementação artificial de *Chilomonas paramecium*. A qualidade nutricional de *C. paramecium* foi elevada através da suplementação com ácido docosahexaenóico (DHA). Os efeitos da suplementação com ácido eicosapentaenóico (EPA) foram mais fracos e não-significantes. Como encontrado anteriormente, os efeitos limitantes só foram

observados com relação a produção de ovos, mas não com o crescimento populacional dos rotíferos.

O presente trabalho fornece evidência de que a composição bioquímica de espécies protistas depende da natureza do recurso alimentar assim como do modo trófico do protista. No entanto, protistas heterotróficos exibiram o potencial para acumular ou até mesmo modificar componentes bioquímicos obtidos em sua dieta. Ainda, a composição bioquímica das espécies protistas analisadas influenciou a reprodução de um predador zooplanctônico. Os resultados aqui apresentados sobre a composição bioquímica em protistas heterotróficos enfatizam a necessidade de se incorporar esses organismos em estudos de qualidade alimentar, devido à sua habilidade em modificar a composição bioquímica em um estágio inicial da cadeia trófica aquática, isto é, na interface localizada entre alga/bactéria e o mesozooplâncton. Tendo em vista o elevado conteúdo energético e o papel essencial de alguns compostos bioquímicos (como lípides), pequenas modificações bioquímicas num estágio inicial da cadeia podem afetar profundamente a transferência de matéria e energia ao longo da cadeia trófica.

1.1 BACKGROUND

1.1.1 Biochemical Composition as Food Quality Parameter in Aquatic Food Webs

It has been recognized for many years that variation in the rate at which primary production is converted to zooplankton biomass is quite large (Sterner and Hessen, 1994). Some aquatic food webs, such as found in many hypereutrophic lakes, have very high biomass of primary producers, but relatively low zooplankton and fish biomasses, as demonstrated by the Eltonian biomass pyramids (Fig. 1). Other systems, such as marine upwelling zones, are low in phytoplankton biomass, but have very high zooplankton and fish biomasses (Fig. 1). This variation in carbon transfer efficiency can be attributed to variation in food quality.

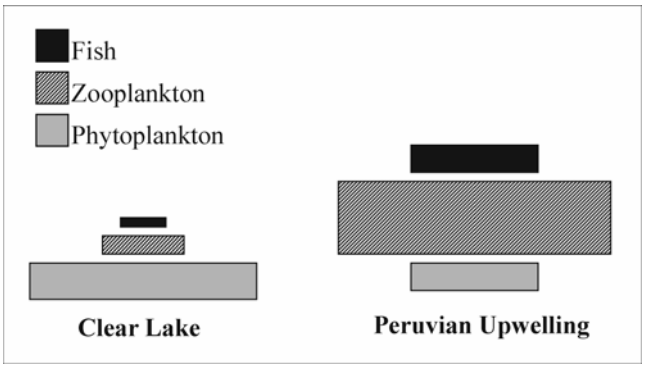

Fig. 1 – Eltonian biomass pyramids for the hypereutrophic Clear Lake (Carney and Goldman, unpublished data) and Peruvian Upwelling Zone food web (Dortch and Packard, 1989). Modified from Brett and Müller-Navarra, 1997.

The food quality of a planktonic prey organism is given by a combination of factors including morphological, physiological, behavioural, chemical, and biochemical features. Additionally, particular requirements of the consumer together with its ability to ingest and digest the prey organism contribute to the determination of the ultimate quality of a planktonic prey. The nutritional value of prey organisms is more specifically determined by the prey mineral (Kilham et al., 1997) and biochemical composition (Kleppel et al., 1998) as well as by the efficiency of a consumer in assimilating prey minerals and essential biochemical compounds (Mayzaud et al., 1992). It is well known, that some cyanobacteria species (blue-green algae), despite their reported edibility for zooplankton in many cases, are a poor food, primarily attributed to phosphorus limitation (Bernardi and Guissani, 1990). On the other hand, phytoplankton species may be neither phosphorus nor nitrogen limited, but lack biochemical compounds, which are essential for herbivorous predators (Müller-Navarra, 1995; Müller-Navarra et al., 2004). Most of the biochemical compounds, which have been considered in

studies of nutritional quality in aquatic food webs, are lipids (fatty acids and sterols) or proteins (especially essential amino acids).

The high-energy content of lipids relative to proteins or carbohydrates coupled with the small body size of most planktonic invertebrates makes lipids the energy storage biomolecules of choice for zooplankton (Arts, 1998). Zooplankton lipids often comprise 60–65% of their dry weight (Arts et al., 1993), and their cellular function depends on the molecular structure (Fig. 2). Triacylglycerols and phospholipids are biochemically related, as they have a glycerol backbone to which two or three fatty acids are esterified (Fig. 2). Triacylglycerols are very important energy storage molecules, whereas phospholipids are essential components of membranes. Sterols share with phospholipids a structural function in membranes, but in terms of polarity they are grouped with triacylglycerols in the neutral lipids. Phospholipids are grouped with polar lipids, including glycolipids. Glycolipids contain one or more molecules of a sugar and are found in bacteria, plants, and animals.

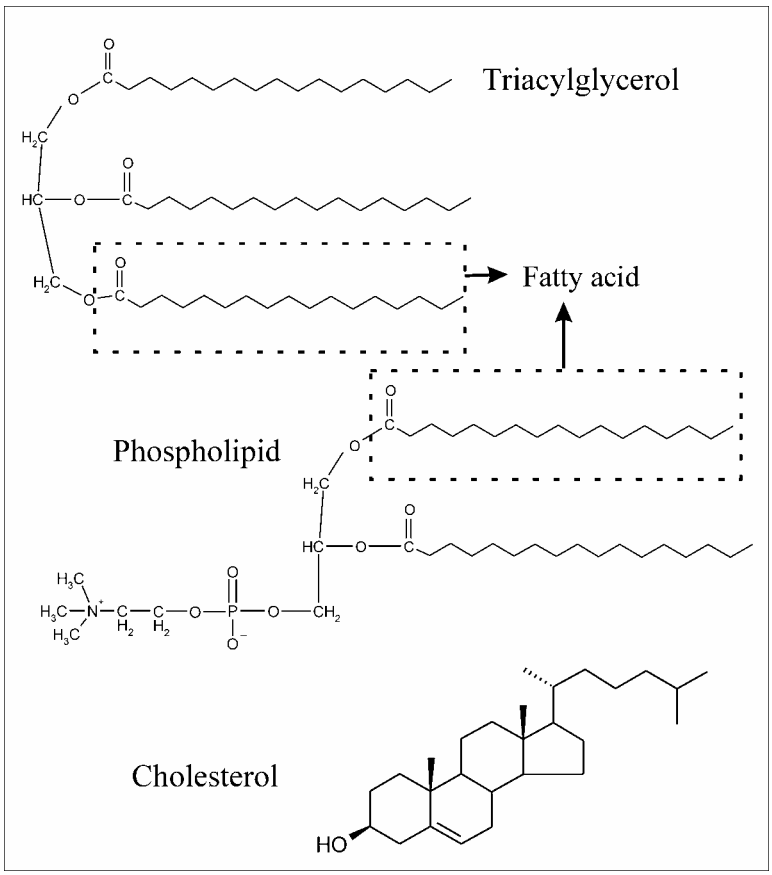

Fig. 2 – The structure of some important lipids (modified from Parrish, 1999).

Among lipid molecules, **fatty acids** have received considerable attention, because they are usually present in low amounts but serve very important physical and metabolic functions in the cell. Especially highly unsaturated fatty acids (HUFA)

of the ω3 and ω6 families, like eicosapentaenoic acid (20:5ω3, EPA) and docosahexaenoic acid (22:6ω3, DHA) as well as some polyunsaturated fatty acids (PUFA), like arachidonic acid (20:4ω6) and linoleic acid (18:2ω6) are considered essential for many zooplanktonic consumers, which are not able to synthesize fatty acids (see appendix 1 for EPA and DHA synthesis and fatty acid nomenclature). Enhanced growth and reproduction of cladocerans, especially *Daphnia*, have been associated with higher contents of HUFA and PUFA in cultured prey organisms (e.g. DeMott and Müller-Navarra, 1997; Park et al., 2002; Becker and Boersma, 2003) and in lake seston (e.g. Müller-Navarra et al., 2000; Park et al., 2003; Müller-Navarra et al., 2004).

Along with essential fatty acids, the **sterol** composition of prey organisms has been reported to be important in limiting zooplankton life history traits (Von Elert et al., 2003; Hasset, 2004). Besides controlling membrane fluidity and permeability, sterols also form sexual hormones, sterol alkaloids, and act as vitamins. Sterol limitation for growth and reproduction of zooplankton predators has been found to be mainly caused by dietary cholesterol shortage, which has been associated with decreased growth of copepods (Hasset, 2004) and cladocerans (Von Elert et al., 2003), and retarded larval development of crustaceans (Teshima, 1991).

Amino acids are the basic structural unit of protein molecules. Some amino acids cannot be synthesized by animal cells at all or only at low rates (Kleppel et al., 1998; Guisande et al., 2000). These amino acids are considered essential and must therefore be supplied in the diet (see appendix 2 for the structure of the essential amino acids considered in this study).

There is an ongoing debate on the factors, which primarily determine the nutritional quality of a planktonic prey for its consumer – the mineral or the biochemical composition. Some authors have advocated the relevance of prey mineral composition and elemental stoichiometry, especially the P:C and N:C ratios, in determining prey nutritional quality, because mineral nutrients usually limit production and growth in nature (e.g. Sterner et al., 1992; DeMott et al., 1998; Plath and Boersma, 2001). Others have emphasized the importance of essential biochemical compounds in promoting enhanced growth and reproduction of zooplankton, based on correlative evidences derived from laboratory and *in situ* studies (e.g. Ahlgren et al., 1990; Brett and Müller-Navarra, 1997; Weers and Gulati, 1997; Becker and Boersma, 2003). Actually, elements may be present in a wide range of biochemical compounds. Therefore, the biochemistry of the

compound in which an element is present dictates how that element is processed by a consumer (Tang and Dam, 1999). Hence, the concept of elemental stoichiometry, although powerful and useful (see Sterner and Elser, 2002), may not be sufficient to the full understanding of the food-related limitation of zooplankton production.

Further, there is a controversy on the question, which biochemical compound is most important in limiting zooplankton life-history traits. Some authors showed evidence for the essentiality of the polyunsaturated fatty acid EPA for *Daphnia* (Müller-Navarra, 1995; Becker and Boersma, 2003), while others did not find any limitation by EPA on daphniids (Von Elert and Wollfrom, 2001; Weiler, 2001) and suggested that sterols may be more important lipids limiting *Daphnia* growth. Moreover, DHA and linoloenic acid have been reported to limit growth and reproduction of zooplankton (Wacker et al., 2002). It must be kept in mind that nutritional quality is not only a matter of prey composition, but also of predator requirements. The fact that EPA was found to limit *Daphnia* growth under a set of conditions does not necessarily imply limitation under different conditions. This does not mean that EPA is not limiting at all, but that its limitation may be modulated by other factors, which were not quantified. Living organisms are actually a complex "package" of nutrients interacting on molecular, biochemical, and physiological levels. Thus, prey organisms should be viewed as a dynamic pool of nutrients rather than as a static source of unaltered composition waiting to be eaten.

1.1.2 The Significance of Heterotrophic Protists in Aquatic Food Webs

It is known that a large fraction of the primary production may not be consumed directly by herbivorous consumers but is channelled through detrital organic matter via bacterial production to phagotrophic microrganisms. This led to the concept of the "microbial loop" (Fig. 3) (Azam et al., 1983) and to the discovery that planktonic food chains include a higher number of trophic levels than hitherto believed (Fenchel, 1988).

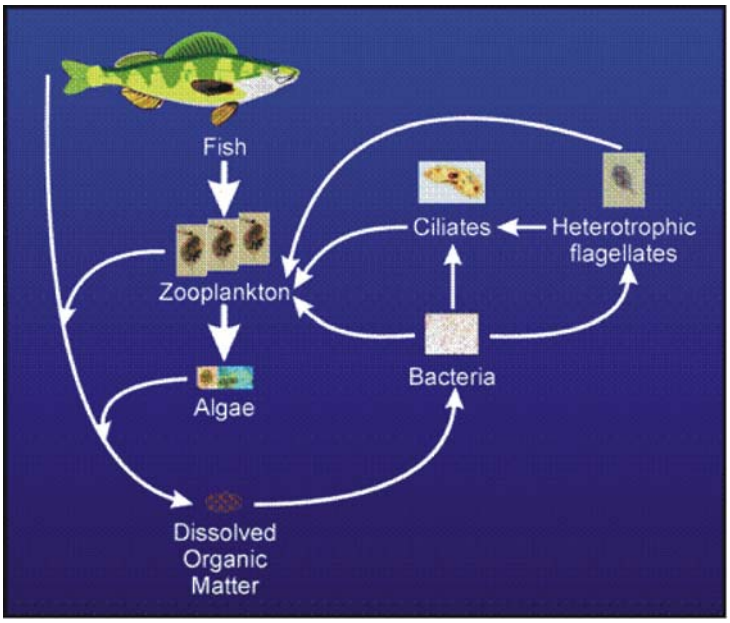

Fig. 3 – Trophic links between the microbial and the classical food webs. Modified from www.biology.ualberta.ca/../energy-flow.htm

However, recent works have shown that the early view of the microbial loop was somehow too simplistic. Today we know that: (1) heterotrophic bacteria do not only rely on dissolved organic matter (DOM) released by autotrophs as a substrate, but also on DOM released by heterotrophs (Jumars et al., 1989); (2) heterotrophic protists comprise more than one trophic level, as predation of ciliates on flagellates is also known (Berninger et al., 1993); (3) grazing by protists may not simply affect bacterial numbers but also have an impact on the morphological structure and productivity of bacterial communities (Jürgens and Güde, 1994); (4) there are immense species-specific differences concerning the trophic roles of protists in a food web; some flagellates, for instance, show autotrophic, mixotrophic and heterotrophic growth under natural conditions (Fenchel, 1986); (5) the production of low trophic levels is not only channelled to higher trophic levels through consumption of bacterial biomass by ciliates and flagellates; but that herbivory by heterotrophic protists also contributes significantly to the matter transfer to higher trophic levels (Sherr and Sherr, 1994). Therefore interactions of heterotrophic protists in planktonic food webs are more various and complex than traditionally believed. The use of simplified "chains" or "loops" does not capture the complexity of microbial food webs (Arndt and Berninger, 1995).

Several laboratorial studies with cultured protists have shown that mesozooplankton predators are able to efficiently grow and reproduce on a diet consisting of heterotrophic protists (Sanders and Porter, 1990; Weisse and Frahm, 2001; Mohr and Adrian, 2001). Moreover, heterotrophic protists are likely to be an important alternative food resource for zooplankton when phytoplankton

abundances are low or when phytoplankton quality is reduced (e.g. during periods of nutrient limitation). High predation pressure by copepods and cladocerans has been observed as a mechanism regulating protist community structure and densities *in situ* (Carrick et al., 1991; Wiackowsky et al., 1994; Jürgens et al., 1999; Jürgens and Jeppesen, 2000; Adrian et al., 2001; Burns and Schallenberg, 2001). However, evidence for high predation rates by mesozooplankton on heterotrophic protists does not attribute them high nutritional quality. Unfortunately, little attention has been paid to the biochemical features of heterotrophic protists, which do confer their nutritional value as prey in aquatic food webs.

1.1.3 The Nutritional Composition of Heterotrophic Protists

Most of the information on the nutritional composition of heterotrophic protists dates back to the 60's and 70's, mostly related to studies of the ciliate *Tetrahymena*. The works of Holz, Connor, and Koroly on *Tetrahymena* were the first to consider the composition and metabolism of lipids and nucleic acids in this ciliate (see Elliott, 1973).

It is well known that heterotrophic protists often have lower C:N ratios than algae and mixotrophic protists (Stoecker and Capuzzo, 1990). The lower C:N ratios suggest that heterotrophic protists may be a source of nitrogen-rich compounds such as amino acids and proteins. Nevertheless, the amino acid composition of free-living heterotrophic protists is virtually unknown, except for studies on *Tetrahymena* (Holz, 1973).

It is has been observed that the fatty acid composition of heterotrophic protists is strongly influenced by the food resources (Ederington et al., 1995; Harvey et al., 1997). However, there are too few studies on the fatty acid composition of heterotrophic protists to permit such a generalization. Indeed, synthesis of long-chain polyunsaturated fatty acids has been reported for marine flagellates (Klein Breteler et al., 1999), suggesting that this metabolic ability should not be excluded for other species.

Despite studies on marine species (Harvey and McManus, 1991; Ederington et al., 1995; Harvey et al., 1997; Breteler et al., 1999; Sul et al., 2000), the sterol composition of freshwater heterotrophic protists is still a 'black box'. The only exceptions are studies on freshwater flagellates of the genus *Chilomonas*, for

which 24α-ethylcholesta-5,22(*E*)-dien-3β-ol (stigmasterol) was described as the predominant sterol (Patterson, 1991), and the bacterivorous ciliate *Tetrahymena pyiriformis*, for which a triterpenoid alcohol – tetrahymanol (gammaceran-3β-ol) – was first isolated and described as its major neutral lipid (Mallory et al., 1963).

It has been observed that the biochemical composition of heterotrophic protists resembles dietary composition (Ederington et al., 1995). For bacterivorous ciliates a fatty acid profile typical for bacteria has been reported, with high concentrations of odd chain-length and branched fatty acids (Ederington et al., 1995). Algivorous protists, in turn, have been reported to show a broader spectrum of polyunsaturated fatty acids, which are not typically found in bacteria (Desvilettes et al., 1997). However, some authors reported the presence of compounds in heterotrophic species, which were not identified in their diet (Ederington et al., 1995; Klein Breteler et al., 1999). The question remains, whether or not heterotrophic protists "are really what they eat", or if they are able to metabolize some compounds obtained from the diet, thus modifying their biochemical composition (heterotrophic protists "are not what they eat").

Some protist species have a fascinatingly complex nutritional ecology. Some flagellated species are able to adapt to a broad range of external conditions, because they exhibit a wide range of trophic modes, such as autotrophy, heterotrophy, and mixotrophy (Sleigh, 2000). Assuming that the trophic mode will ultimately determine the nutritional composition of such species, a large variability in the biochemical composition is expected to be found in these protists. As autotrophs, they are able to synthesize a broad palette of compounds, which are necessary for growth and maintenance. As heterotrophs, they ingest particulate material (phagotrophy) or absorb (osmotrophy) a wide diversity of molecules from their environment, thus extracting organic and inorganic building blocks for their own biosynthesis reactions as well as energy for growth and maintenance (Sterner and Elser, 2002). As mixotrophs, phagotrophy or osmotrophy supplements photosynthesis to generate energy and provide carbohydrate building blocks (Sanders, 1991).

The nutritional complexity of protists raises interesting questions about the nature of the mechanisms, which influence their biochemical composition, and subsequent nutritional quality as prey. Does the biochemical composition of protists depend only on their trophic mode? And within the same trophic mode, e.g. heterotroph/phagotroph, are there differences between algivores and bacterivores, i.e. does the dietary composition determine protist biochemical

composition? Between two algivores fed the same algae, are there species-specific differences in the biochemical composition? Those questions should be answered before one decides to evaluate the nutritional quality of protists for zooplankton predators. Only by knowing the biochemical composition of protists and to which extent it can be dictated by dietary composition or by the trophic mode, it is possible to evaluate what really determines the nutritional quality of protists as prey for planktonic predators.

1.2 Outline Of The Thesis And Hypotheses

This thesis is based on the four following questions treated in form of 5 chapters:

1. Does the biochemical composition of free-living protists depend on their dietary sources? **chapters 2 and 3**
2. Does the biochemical composition of protists depend on the trophic mode? **chapter 4**
3. Does the biochemical composition of heterotrophic protists determine their nutritional quality for the rotifer *Keratella quadrata*? **chapter 5**
4. Does the supplementation of *Chilomonas paramecium* with essential fatty acids enhance their nutritional value for *Keratella quadrata*? **chapter 6**

To answer the first question I investigated the fatty acid, amino acid, and sterol composition of four phagotrophic protist species and their diet: the algivorous ciliates *Balanion planctonicum* and *Urotricha farcta* were fed the cryptomonad *Cryptomonas phaseolus*; the bacterivorous ciliate *Cyclidium* sp. and the flagellate *Chilomonas paramecium* were fed bacteria grown on rice corns (Fig. 4).

The second question was addressed by analysing the fatty acid and sterol composition of the flagellate *Ochromonas* sp. cultured under different trophic modes – autotrophic, mixotrophic, and heterotrophic (Fig. 4).

To evaluate the influence of the biochemical composition of heterotrophic protists on their nutritional quality as prey I performed population growth and reproduction experiments using the rotifer *Keratella quadrata* as a predator (Fig. 4). I chose *K. quadrata* as a model predator, because (1) of the ecological significance of this species in many lakes as a potential predator of heterotrophic protists and because of (2) the lack of knowledge concerning the nutritional value of heterotrophic protists for rotifers. The rotifer was offered the four protist

species analysed in chapters 2 and 3, and the biochemical composition of the protists was correlated to population growth rates and to the cumulative number of eggs produced by *K. quadrata* during the experiments.

The last question was formulated because I found evidence for the potential of some biochemicals of the protists to limit egg production of the rotifers. To test the effect of single biochemical compounds directly I artificially supplementated one protist species – *Chilomonas paramecium* – with two selected polyunsaturated fatty acids (EPA and DHA) and evaluated the effect of supplemented versus non-supplemented flagellates on population growth and egg production of *K. quadrata* (Fig. 4).

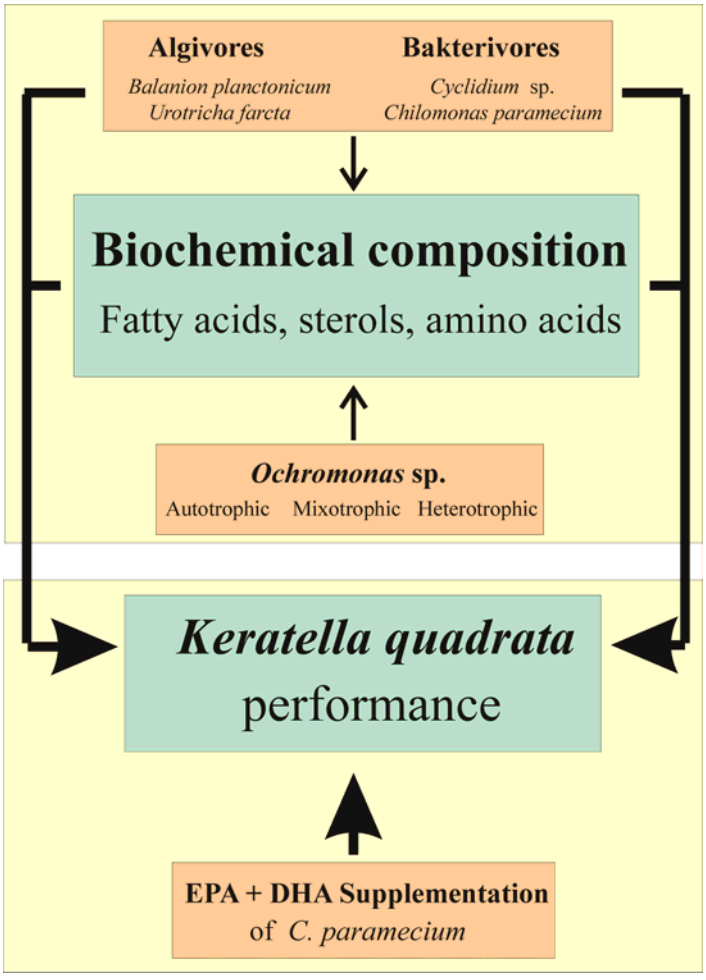

Fig. 4 – Outline of the thesis. Upper box: Experiments related to the influence of dietary composition (chapters 2 and 3) and trophic mode (chapter 4) on the biochemical composition of protists. Lower box: Experiments related to the nutritional value of heterotrophic protists for *Keratella quadrata* (chapters 5 and 6).

The following hypotheses were tested:

Hypothesis 1. Dietary biochemical composition determines the biochemical composition of heterotrophic protists.

To address this hypothesis, I selected four species, which were fed different diets: the algivorous ciliates *Balanion planctonicum* and *Urotricha farcta* were fed the cryptomonad *Cryptomonas phaseolus*. The ciliate *Cyclidium* sp. and the flagellate *Chilomonas paramecium* were fed bacteria grown on rice corns. The fatty acid and amino acid composition (chapter 2) as well as the sterol composition (chapter 3) were analysed in both the protists and their diet. Discrepancies between protist and dietary biochemical composition are discussed in light of metabolic features of the protists. Differences between two species fed the same diet are discussed in terms of species-specific metabolic features of the protists.

Hypothesis 2. The trophic mode determines the biochemical composition of the flagellate *Ochromonas* sp.

To test this hypothesis, the fatty acid and sterol composition of the flagellate *Ochromonas* sp. grown as autotroph, mixotroph, and heterotroph was analysed (chapter 4). The role of photosynthesis and phagotrophy in determining metabolic patterns of fatty acid and sterol synthesis and accumulation are discussed.

Hypothesis 3. Heterotrophic protists fed different diets have different nutritional quality for a rotifer predator due to differences in their biochemical compounds.

To test this hypothesis, the rotifer *K. quadrata* was separately offered two algivorous and two bacterivorous protists as prey (chapter 5). The cryptomonad *C. phaseolus* was used as a good quality control food. The biochemical composition of the protists, which was analysed in the previous chapters, was correlated with population growth rates and the egg numbers of the rotifer.

Hypothesis 4. *Chilomonas paramecium* can be sucesssfully supplemented with essential fatty acids (EPA and DHA), in order to test the influence of these biochemicals on rotifers' life-history traits.

Correlation analyses between prey biochemical composition and the cumulative number of eggs produced by the rotifer *K. quadrata* (see chapter 5), provided evidence for limiting effects of EPA and DHA, among other compounds. I tested a new supplementation technique (Von Elert, 2002) for supplementing the heterotrophic flagellate *Chilomonas paramecium* with EPA and DHA (chapter 6).

Up to now, this supplementation technique has been only used for supplementing algal cells. The efficiency of the method was tested by supplementing *C. paramecium* with different EPA and DHA incubation concentrations. To test whether the nutritional quality of *C. paramecium* was enhanced through EPA and DHA supplementation, the effects of supplemented versus non-supplemented *Chilomonas* on population growth rates and egg numbers of the rotifer *K. quadrata* were evaluated.

2. BIOCHEMICAL COMPOSITION OF FRESHWATER HETEROTROPHIC PROTISTS: DOES IT DEPEND ON DIETARY COMPOSITION?

Iola G. Boëchat and Rita Adrian

(*FEMS – Microbiology Ecology*, under revision)

Keywords: Heterotrophic protists; Biochemical composition; Fatty acids; Amino acids; Dietary composition

ABSTRACT

The focus of our study was to determine whether the biochemical composition of heterotrophic protists resembles that of their diet. Carbon- and cell-specific concentrations of fatty acids and essential amino acids were investigated for two ciliates (*Balanion planctonicum*, *Urotricha farcta*) grown on algal diet (the cryptomonad *Cryptomonas phaseolus*), and a ciliate and a flagellate (*Cyclidium* sp. and *Chilomonas paramecium*) grown on a mixed diet consisting of bacteria and small rice particles. Stepwise discriminant analyses (SDA) indicated differences in the fatty acid and amino acid composition between heterotrophic protists and their diet, as well as among protist species. Carbon-specific fatty acid and amino acid concentrations were usually higher in the heterotrophic protists than in their diet. *B. planctonicum* and *U. farcta* showed higher concentrations of monounsaturated and some polyunsaturated fatty acids than their algal diet. Moreover, except for tryptophan, valine, and lysine, higher carbon-specific amino acid concentrations were observed in both *B. planctonicum* and *U. farcta* than in *Cryptomonas*. *Cyclidium* sp. and *C. paramecium* had higher carbon-specific concentrations of polyunsaturated fatty acids and amino acids than their diet, except for histidine, methionine, and leucine. Cellular-specific fatty acid concentrations were generally higher in the protists fed the algae than in the protists fed bacteria and rice particles, while cellular-specific amino acid concentrations were similar among protists. The higher fatty acid and amino acid concentrations in the heterotrophic protists compared to their diet may suggest that these species are capable of efficiently assimilating or even synthesising biochemical compounds. We conclude that dietary fatty acid and amino acid composition influences the composition of the four freshwater protist species to a minor extent, and that species-specific differences in fatty acid and amino acid metabolism are more important determinants of the biochemical composition in the analysed heterotrophic protists.

2.1 INTRODUCTION

Aquatic food webs rely on autotrophs as these are the most important resource of essential chemical components for the mesozooplankton. Heterotrophic protists on the other hand are an important trophic link in aquatic food webs, as they prey on primary producers and bacteria, and are themselves preyed upon by the mesozooplankton (De Biase et al., 1990; Wickham et al., 1993; Sanders et al., 1996; Adrian and Schneider-Olt, 1999; Adrian et al., 2001; Burns and Schallenberg, 2001; Mohr and Adrian 2002a). Depending on their ability to assimilate and incorporate chemical compounds obtained from their diet, heterotrophic protists have the potential to modify the chemical composition of organic matter at an early stage in the food chain.

In zooplankton species, the fatty acid composition of a predator usually resembles that of its prey (Ederington et al., 1995; Fernández-Reiriz and Labarta, 1996; Von Elert and Stampfl, 2000). *Daphnia galeata* exhibited higher concentrations of the polyunsaturated fatty acids EPA (eicosapenenoic acid) and DHA (docosahexaenoic acid) when fed a cryptomonad than *D. galeata* fed green algae (Weers et al., 1997). Similarly, *Acartia tonsa* exhibited higher concentrations of saturated and monounsaturated fatty acids when fed a bacterivorous ciliate and higher concentrations of polyunsaturated fatty acids when fed diatoms (Ederington et al., 1995). A study involving two marine ciliates (Harvey et al., 1997) showed that the lipid composition (fatty acids, neutral lipids, and sterols) of the ciliates resembled that of their prey (either bacteria or algae). Assuming that these findings are also valid for freshwater heterotrophic protists, one would expect differences in the chemical make-up of heterotrophic protists fed different diets. Indeed, bacterivorous protists contained large amounts of saturated (SAFA) and monounsaturated (MUFA) fatty acids (together comprising more than 85% of the total fatty acids), and very low quantities of polyunsaturated fatty acids (PUFA), and highly unsaturated fatty acids (HUFA) (Ederington et al., 1995). In contrast, algivorous ciliates are expected to contain high quantities of PUFA, HUFA, and some essential amino acids (Desvilettes et al., 1997). In algivorous marine ciliates, SAFA and PUFA comprised 32% and 57% of the total fatty acids (Klein Breteler et al., 1999). The amino acid composition of mesozooplankton predators seems to be rather constant, and more or less independent of the amino acid composition of their diet (Frolov et al., 1991; Cowie and Hedges, 1994; Guisande et al., 1999; Guisande et al., 2000; Helland et al., 2003a,b). Except for

studies on *Tetrahymena* (Holz, 1973), the amino acid composition of freshwater heterotrophic protists is virtually unknown.

Different protist species may show different metabolic features. To date little is known about species-specific differences in fatty acid and amino acid metabolism of freshwater heterotrophic protist species. Such differences are expected to occur and may contribute to the transfer of essential compounds between primary producers and higher level consumers in aquatic food webs. In addition to the assimilation and incorporation of dietary compounds, *de novo* synthesis of some essential compounds has been observed in ciliates and flagellates. The bacterivorous ciliate *Pleuronema* sp. was found to contain high concentrations of a triterpenoid alcohol (tetrahymanol), as a major neutral lipid, which was not observed in its bacterial prey (Ederington et al., 1995). Also the ability of the heterotrophic dinoflagellate *Oxyrrhis marina* to synthesize EPA, DHA, and some sterols has already been described (Klein Breteler et al., 1999).

We now address the question of how the biochemical profile (fatty acids and essential amino acids) of freshwater heterotrophic protists reflects that of their diet. Furthermore, we looked for differences in the biochemical composition between protist species fed the same diet. To elucidate the relationship between the biochemical composition of heterotrophic protists and their diet, we compare the fatty acid and essential amino acid composition of four protist species with the fatty acid and essential amino acid composition of their diet. The ciliates *Balanion planctonicum* and *Urotricha farcta* were cultured on the cryptomonad *Cryptomonas phaseolus* and the ciliate *Cyclidium* sp. and the flagellate *Chilomonas paramecium* were cultured on a mixed diet consisting of bacteria and small rice particles. Our study examines the fate of the biochemical compounds, which confer food quality to heterotrophic protists and emphasizes species-specific differences in protist ability to transform energy and organic matter at an early stage in the aquatic food web, when the disparity in the biochemical composition may be large.

2.2 METHODOLOGY

2.2.1 Cultures

The ciliates *Balanion planctonicum* WULLF, 1922 (average biovolume 3256 ± 1331 μm^3) and *Urotricha farcta* CLAPARÈDE and LACHMANN, 1858 (2778 ± 1707 μm^3) were cultured in WC medium (Guillard and Lorenzen, 1972) in frequently diluted batch cultures incubated at 17°C under a 12:12 h light : dark regime. The ciliates were fed the cryptomonad *Cryptomonas phaseolus* EHRENBERG, 1832 (392 ± 125 μm^3), obtained from the Algal Collection of the University of Göttingen, Germany, and cultured in WC medium at 17 ± 1°C under a 16:8 h light : dark regime. Our cultures were non axenic, but bacteria accounted for less than 2 % of the total organic carbon contents in the cultures of the protists and *C. phaseolus*. Although these ciliates are also known to ingest bacteria, especially *U. farcta* (Foissner et al., 1999), we consider these ciliates as algivores, as they were mainly fed an algal prey. According to microscopic observations, they indeed preyed upon the algae, which were reduced to less than 10% of the total carbon content of the cultures, usually within a five days growth period. The flagellate *Chilomonas paramecium* EHRENBERG, 1832 (403 ± 288 μm^3) and the ciliate *Cyclidium* sp. (1315 ± 617 μm^3) were cultured in Volvic water (a spring water, poor in minerals, sold worldwide by Société des Eaux de Volvic, Puy-de-Dôme, France) at 18 ± 1°C under a 16:8 h light : dark regime. Both were cultured on a mix of bacteria grown on previously autoclaved polished rice corns (referred to as "bacteria + rice" diet, since the protists might have been able to ingest rice particles along with bacteria in the medium). Although the contribution of small rice particles to the total carbon biomass in the cultures was small compared to bacterial biomass (based on microscopic observations), the influence of the biochemical composition of rice should not be ignored, and it has to be considered when discussing the results. Henceforth, species are referred to by their genus names only.

2.2.2 Sample Preparation and Extraction

Samples of heterotrophic protists and the algal diet were taken from the cultures, fixed with Lugol's solution and counted under a stereomicroscope at x 20 magnification. For bacteria enumeration, 1 mL samples were fixed in 2% formaldehyde, filtered onto 0.2 μm pore size black polycarbonate membranes, stained with DAPI (4,6–diamidino–2–phenylindol) (Porter and Feig, 1980) and

counted under an epifluorescence microscope (excitation wavelength 450–490 nm combined with an FT 510 beam splitter and a LP 520 suppression filter). Carbon concentrations in heterotrophic protists and their diet were derived from cell volume estimates using carbon : biovolume conversion factors of 0.10 pgC μm^{-3} for the alga *Cryptomonas* (Montagnes et al., 1994), 0.19 pgC μm^{-3} for heterotrophic protists (Putt and Stoecker, 1989), and 0.125 pgC μm^{-3} for the bacteria (Pelegrí et al., 1999). Cell biovolumes of the protists, algae, and bacteria were estimated based on commonly used geometric forms (sphere, cylinder and ellipsoid). Unfortunately, we were not able to separate the algivorous ciliates from their algal diet via filtration or centrifugation due to their similar cell dimensions – an inherent problem in working with micrograzers. To minimize this problem we collected samples for the biochemical analyses at the end of the exponential growth phase, when the algivorous ciliates had diminished *Cryptomonas* to 4–16% of the total organic carbon content of the cultures. To a lesser extent, this was also the case for the heterotrophic protists growing on bacteria and rice corns, although they were previously filtered successively over a 10 μm and a 5 μm mesh. Due to this previous filtration, bacterial biomass was reduced to less than 20% of the total bacterial carbon content in the cultures. The filtration procedure also reduced the occurrence of large rice particles in the samples. However, rice particles and bacterial cells smaller than 5 μm were still present in the samples for biochemical analyses. Since a complete separation of heterotrophic protists and their diet was not possible, we adopted a subtraction method in order to estimate the fatty acid and amino acid composition and concentration in heterotrophic protists separately. By knowing the carbon-specific fatty acid and amino acid contents of the diets, obtained from analysis of either the algal or "bacteria + rice" sole-cultures, we determined the biochemical concentration of the heterotrophic protists indirectly through subtraction. For the biochemical analyses, 200 to 300 mL of each culture were filtered in 2–3 replicates on pre-incinerated (550°C, 4 h) GF/C Whatman glass fiber filters. Samples of bacterial cultures were collected on GF/F Whatman glass fiber filters. Samples had a minimum concentration of 1–3 mgC. The analyses were repeated for 4–8 different batches of each consumer-diet combination and the sole dietary cultures. We collected protist cells at the end of the exponential phase because algal and bacterial prey densities were minimal in the cultures by this time (see sample preparation above). Nevertheless, protist cells were still within the exponential growth phase, during which food quality has been found to be the highest (Sterner, 1993). Filters containing the material for the fatty acid analyses were immediately extracted with chloroform-methanol 2:1, v/v (Folch et al., 1957) and homogenised by sonification for 5 min at 5000 cycles min^{-1} (Ultrashall-Desintegrator USD 20, VDE Wiss. Gerätebau, Berlin).

Cryptomonas samples, however, were submitted to a 10 min sonification time period, which assured cell destruction. After homogenising, an internal standard was added to the samples (tricosanoic acid, 0.2 mg mL^{-1}). The samples were allowed to extract for 3 h, at 20°C. After extraction, samples were dried under nitrogen flux and promptly stored at –20°C until analysis. Samples for amino acid analyses were lyophilized and promptly stored at –20°C pending further analysis.

2.2.3 Fatty Acid Analyses

Fatty acid methyl-esters (FAME) were formed by addition of 5 mL sulphuric acid (5% v/v) and heating the samples for 4 h at 80°C (Weiler, 2001). An aliquot of 0.2 µL of the samples was finally injected into a Varian Star 3600 CX series gas chromatograph, equipped with a fused silica capillary column (Omegawax 320, SUPELCO, 30 m x 0.32 mm). The following heating program was applied: initial temperature of 180°C (2 min), subsequent heating at 2°C min^{-1} to 200°C, which was held isothermally for 33 min Injector and FID detector temperatures were 250°C and 260°C, respectively. Helium was used as a carrier gas. FAMEs identification were made by comparing the retention times with retention times of a calibration standard solution (Supelco FAME Mix 47885–4, PUFA Nr. 3–47085–4 and PUFA Nr. 1–47033) and quantified by comparing the peak areas with the peak area of the internal standard. For presentation, we selected some fatty acids from the total measured pool. It should be kept in mind that the sums of saturated, monounsaturated, and polyunsaturated fatty acids presented in the results refer to the whole pool of measured fatty acids.

2.2.4 Amino Acid Analyses

Lyophilized filters were hydrolysed by the addition of 6N HCL and incubation of the samples at 110°C for 24 h. After hydrolysis, samples were neutralized with 6N NaOH. A 200 µL aliquot of the neutralized amino acid solution was diluted with 1 mL of a methanol : water (80:20, v/v) solution (Ogunji and Wirth, 2001). A 50 µL internal standard (4 µg mL^{-1} homoserine) was added to an aliquot of 500 µl of the diluted samples and the vials were transferred to an Auto Sampler. A 50 µL aliquot of the diluted mixture was injected in triplicate into a Merck/Hitachi HPLC system (Ogunji and Wirth, 2001) using a Nova-Pak C18 column, 4 µm, 3.9 x 300 mm (Waters GmbH, Germany).

2.2.5 Statistical Analyses

Differences in the fatty acid and amino acid composition between heterotrophic protists and their diet were tested using one-way ANOVA, followed by Dunnett's

test. In this case, the biochemical composition of the protists was tested against dietary biochemical composition, used as experimental control for this purpose. Pairwise comparisons of the biochemical composition among protist species were performed with a Tukey *HSD* – test following one-way ANOVA. To test the hypothesis that fatty acid and amino acid composition in the protists was dependent on dietary composition, we performed stepwise discriminant analyses (SDA). Discriminant analysis is used to determine which variables better discriminate between two or more defined groups. In our case, the analyses were performed to determine which fatty acids and amino acids (variables) better separated the organisms we analysed (groups). The basic idea underlying the discriminant analysis is to determine whether groups differ with regard to the mean of a variable, and then to use that variable to predict group membership. In the case of a multiple group stepwise discriminant analysis (6 different groups – 4 protist species and two diets in our analysis), the ultimate calculations correspond to a canonical correlation analysis, which provides the successive functions and canonical roots containing the variables mostly responsible for separating the groups. For the fatty acid data, SDA were performed separately for saturated, monounsaturated, and polyunsaturated fatty acids. Although we do not present the entire pool of fatty acids we measured, all fatty acids were considered for the statistical analyses. We performed one SDA for the whole pool of amino acid data. All statistical procedures were run in Statistica for Windows (version 5.01, Stat Soft).

2.3 **RESULTS**

2.3.1 Fatty Acids

We found higher amounts of saturated and polyunsaturated than of monounsaturated fatty acids in *Cryptomonas* (Table 1). High concentrations of the saturated acids 16:0 and the polyunsaturated acids 18:3ω3 and 18:4ω3, as well as high concentrations of eicosapentaenoic acid (EPA) for *Cryptomonas* sp. have already been described (Ahlgren et al., 1990; Ahlgren et al., 1992; Von Elert and Stampfl, 2000). Our *Cryptomonas* species contained also high carbon-specific concentrations of linoleic acid (18:2ω6), 20:(2–3)ω6, arachidonic acid (20:4ω6), docosapentaenoic acid (DPA, 22:5ω3), and docosahexaenoic acid DHA (22:6ω3).

The composition of all major fatty acid classes (SAFA, MUFA, PUFA) were similar in the two ciliates fed *Cryptomonas* and basically resembled the composition of the algal diet (Table 1). The only exceptions were DPA and DHA, which were not found in the ciliates, yet present in *Cryptomonas*, and the polyunsaturated fatty acid 20:3ω6, which was not found in *Balanion* (Table 1). However, carbon-specific concentrations of almost all monounsaturated and some polyunsaturated fatty acids were higher in the ciliates than in their algal diet (Dunetts' test, $P<0.05$; Table 1). *Cryptomonas* contained more ω6 than ω3 fatty acids, while both *Balanion* and *Urotricha* showed the inverse pattern, as shown by the ω6:ω3 ratios (Table 1). Total SAFA, MUFA, and PUFA concentrations in *Balanion* and *Urotricha* were similar, although concentrations differed for a few fatty acids (e.g. DHA, Tukey *HSD* – test, $P<0.05$).

A fatty acid distribution typical of bacteria was observed in the "bacteria + rice" diet (Table1). High carbon-specific concentrations of saturated fatty acids such as 16:0, 18:0, 19:0, and 24:0 were found, in agreement with other studies (Fredrickson et al., 1986; Kaneda, 1991; Harvey et al., 1997). We also found high carbon-specific concentrations of the monounsaturated fatty acids 16:1ω9 and 16:1ω5 both considered typical for bacteria (Gillan et al., 1981). However, we observed high carbon-specific concentrations of polyunsaturated fatty acids, in contrast with previous studies (Kaneda, 1991; Harvey et al., 1997).

The fatty acid composition of *Cyclidium* and *Chilomonas* deviated more considerably from that of their "bacteria + rice" diet (Table 1). Although not present in their "bacteria + rice" diet, the fatty acids 14:0, 18:3ω6, 20:2ω6, 20:3ω6, and 21:5ω3 were found in both *Cyclidium* and *Chilomonas* (Table 1). Higher SAFA, MUFA, and PUFA carbon-specific concentrations were observed in the heterotrophic protists than in their "bacteria + rice" diet, except for the saturated acid 24:0, which was found in very low concentrations in the protists, despite its elevated concentration in the "bacteria + rice" diet (Dunnetts' test, $P<0.05$). On the other hand, the monounsaturated acid 20:1ω9 was found in high carbon-specific concentrations in the "bacteria + rice" diet and in *Cyclidium*, but could not be detected in *Chilomonas* (Table 1). We detected DHA in *Chilomonas*, although not present in the "bacteria + rice" diet and in *Cyclidium* (Table 1). Carbon-specific concentrations of monounsaturated fatty acids in *Cyclidium* were higher than those found in *Chilomonas*, largely attributable to discrepancies in the concentrations of 18:1ω7 and 20:1ω9 (Table 1). *Cyclidium* contained higher EPA concentrations than *Chilomonas* and the algal diet *Cryptomonas* (ANOVA, and subsequent Tukey *HSD* – test, $P<0.01$; Table 1).

Balanion and *Urotricha* had significantly higher carbon-specific concentrations of SAFA ($P<0.001$), MUFA ($P<0.001$), and PUFA ($P<0.01$) than either *Cyclidium* or *Chilomonas*, or both (ANOVA, and subsequent Tukey *HSD* – test). Carbon-specific EPA concentrations were similar among *Balanion*, *Urotricha*, and *Cyclidium*, and were higher than in *Chilomonas* (ANOVA, and subsequent Tukey *HSD* – test, $P<0.01$; Table 1). These results are in agreement with the significantly higher total carbon-specific fatty acid concentrations observed in the algal diet *Cryptomonas* than in the "bacteria + rice" diet, except for monounsaturated fatty acids (Table 1).

Stepwise discriminant analyses for fatty acids were run separately for saturated, monounsaturated, and polyunsaturated fatty acids. Although the analyses often provided more than two significant canonical roots (discriminant functions) only the first two roots are presented in each case (Fig. 1), because together they explained more than 85% of the total variation among groups. The results of the first 3 roots are presented in Table 2.

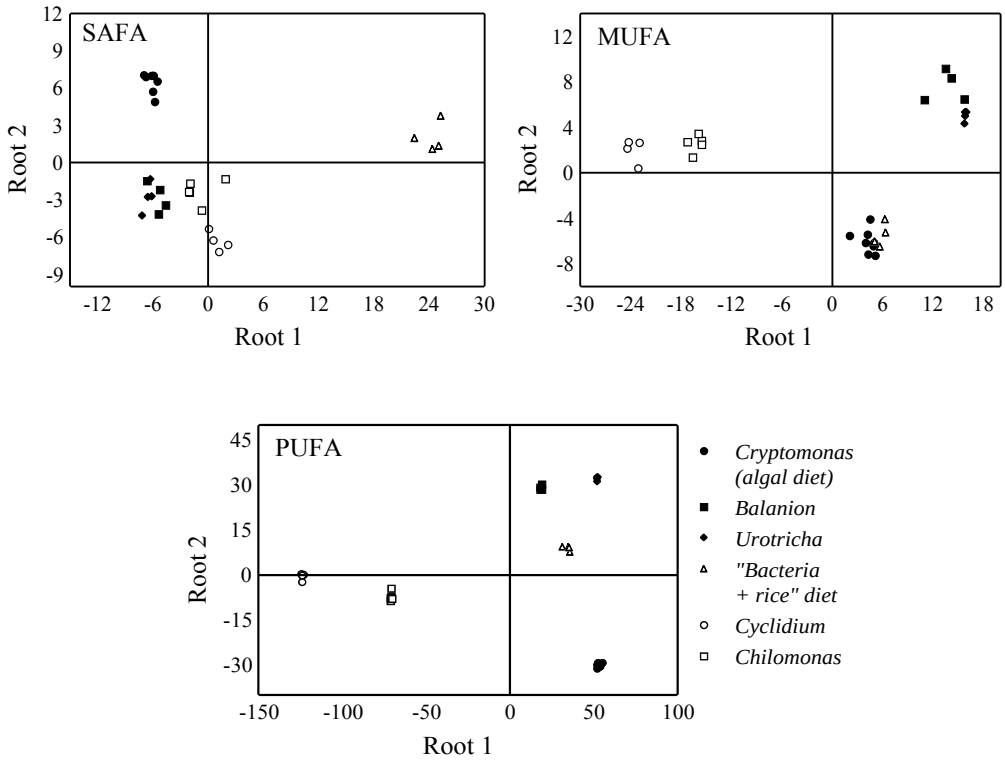

Fig. 1 – Canonical roots provided by stepwise discriminant analyses (SDA) run separately for saturated (SAFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA). See text and Table 2 for the fatty acids discriminating within each root.

The SDA on saturated fatty acids provided 4 significant roots, each root representing one or more fatty acids responsible for discriminating between protists and dietary assemblages (Wilks' Lambda for SDA<0.0001, approximated F=25.1, *P*<0.001). The "bacteria + rice" diet was separated from all other organisms at the first root, due to their significant lower 20:0 concentrations and their higher 24:0 concentrations (Fig. 1, Table 2). Three groups were discriminated at the second root, basically due to the significant differences in the concentrations of the saturated fatty acids 12:0, 19:0, and 21:0 (Fig. 1, Table 2). *Cryptomonas* was separated from *Balanion* and *Urotricha*, which formed a second group together with *Chilomonas* and the "bacteria + rice" diet. The third group discriminated at this root was composed only by *Cyclidium*. The third root discriminated between *Urotricha* and *Balanion*, due to the significant differences in the saturated acids 8:0 and 15:0 (Table 2). *Cyclidium* and *Chilomonas* were better separated at the fourth root of the SDA, basically due to the differences in the saturated fatty acid 10:0.

The second SDA, based on differences in monounsaturated fatty acids between protists and diets, provided 4 significant canonical roots (Wilks' Lambda for SDA<0.0001, approximated F=16.5, *P*<0.001). The first root discriminated among three groups as follows: the first group was formed by *Chilomonas* and *Cyclidium*, the second by both the algal and "bacteria + rice" diets, and the third one was formed by the ciliates fed the algal diet (Fig. 1). Especially the significant differences involving the monounsaturated fatty acids 18:1ω7, 22:1ω11, and 16:1ω5 were responsible for discriminating between these groups (Table 2). The second root more efficiently separated diets from protists (Fig. 1) basically due to the significant differences involving the monounsaturated fatty acids 12:1, 14:1ω5, and 16:1ω7 (Table 2). *Cyclidium* and *Chilomonas* were separated at the third root, due to the significant differences involving the 20:1ω9 (Table 2). The two ciliates fed the algae were separated at the fourth root, due to the differences in the concentrations of 18:1ω9. Given their similar concentrations of monounsaturated fatty acids, the algal and the "bacteria + rice" diets were not discriminated by any root provided by the SDA on monounsaturated fatty acids.

The SDA based on polyunsaturated fatty acids provided the best discrimination among groups, resulting in three significant roots (Wilks' Lambda for SDA<0.0001, approximated F=35.9, *P*<0.001). The first root discriminated between two groups. The first group was composed by *Cyclidium* and *Chilomonas*, the second one by *Urotricha*, *Balanion*, the algal diet *Cryptomonas*, and the "bacteria + rice" diet (Fig. 1). Moreover, a slight separation was observed between *Cyclidium* and *Chilomonas* at this root. Overall, differences in the

polyunsaturated fatty acids 22:3ω6, 18:2ω6, 22:5ω3, 18:3ω6, 20:2ω6, 20:4ω3, and 18:3ω3 contributed most to the separation observed at this root (Table 2). The second root separated *Balanion* and *Urotricha* from their algal diet *Cryptomonas* (Fig. 1). The algal diet was also separated from the "bacteria + rice" diet at this root. The polyunsaturated fatty acids separating groups within this root were 22:2ω6, DPA, DHA, 16:3ω4, 16:2ω4, and 20:3ω6. The third root efficiently separated *Balanion* from *Urotricha*, and *Cyclidium* from *Chilomonas*, due to the significant differences observed for 20:3ω3, EPA, and 18:3ω4 (Table 2).

2.3.2 Amino Acids

All essential amino acids found in the diet were also found in the heterotrophic protists (Table 3). However, the carbon-specific amino acid concentrations differed between protists and diet, as revealed by the Dunnett's tests following one-way ANOVA (Table 3, $P<0.05$). *Balanion* and *Urotricha* showed higher carbon-specific concentrations than their algal diet *Cryptomonas* for 6 of 10 amino acids (threonine, arginine, methionine, phenylalanine, isoleucine, and leucine; Table 3). Moreover, *Balanion* had higher carbopn-specific histidine concentrations than *Cryptomonas*, whereas *Urotricha* had higher tryptophan and lysine concentrations than the algal diet (Table 3). Only valine concentrations did not differ significantly between both ciliates and their algal diet *Cryptomonas*. Except for leucine and tryptophan, carbon-specific amino acid concentrations were similar in *Balanion* and *Urotricha* (Tukey *HSD* – tests, $P<0.05$ for leucine and tryptophan).

Except for histidine, methionine, and leucine concentrations, the carbon-specific amino acid concentrations were significantly higher in both *Cyclidium* and *Chilomonas* than in their "bacteria + rice" diet (Table 3). Moreover, higher leucine concentrations were found in *Cyclidium*, and higher histidine and methionine concentrations were found in *Chilomonas* than in the "bacteria + rice" diet (Dunnetts' test, $P<0.05$). *Chilomonas* had significantly higher carbon specific histidine, arginine, tryptophan, valine, isoleucine, and lysine concentrations than *Cyclidium* (Tukey *HSD* – test, $P<0.01$), although both species were fed the same diet (Table 3).

The SDA provided 5 discriminant functions (given by canonical roots), but only the first two were significant (Wilks' Lambda for SDA=0.00007, approximated $F=7.96$, $P<0.001$) (Table 4). The first canonical root separated a group composed by *Balanion*, *Urotricha*, and *Chilomonas*, from a group formed by *Cyclidium* and both diets (Fig. 2). Phenylalanine, lysine, tryptophan, methionine, isoleucine, and

valine were the amino acids separating the groups at this root. The second root separated *Cyclidium* from the dietary assemblages (Fig. 2), mainly due to differences in histidine and leucine concentrations (Table 4). According to the SDA no significant differences were detected for the amino acid concentrations between the algal and the "bacteria + rice" diet.

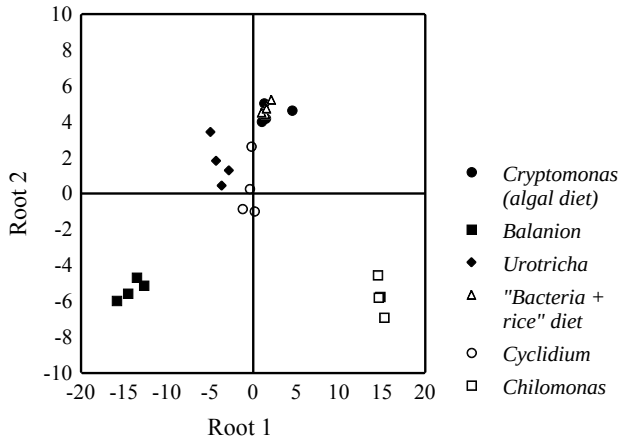

Fig. 2 – Canonical roots provided by stepwise discriminant analysis (SDA) for amino acid data. The first root separated *Chilomonas*, *Balanion*, and *Urotricha*. Second root separated *Cyclidium* from both diets. See text and Table 4 for the amino acids discriminating within each root.

2.3.3 Cell-Specific Concentrations of Biochemical Compounds

We found a positive correlation between cell-specific fatty acid concentration and cell size. The algivores *Balanion* (3256 μm^3) and *Urotricha* (2778 μm^3) had significantly higher concentrations of saturated, monounsaturated, and polyunsaturated fatty acids, including EPA (Tukey *HSD* – Test, all *P* values <0.001) than *Cyclidium* (1315 μm^3) and *Chilomonas* (403 μm^3) (Fig. 3). Cell-specific amino acid concentrations were similar in all protist species, except for threonine ($P<0.01$) and leucine ($P<0.001$), which were significantly higher in *Balanion* (Fig. 3).

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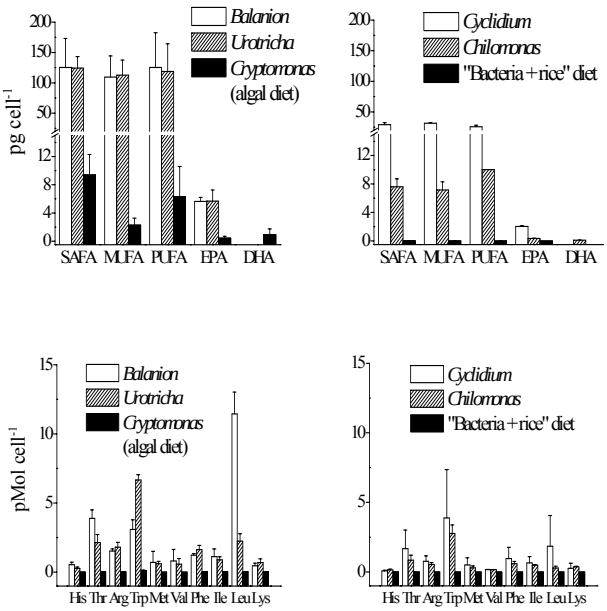

Fig. 3 – Cell-specific concentrations of fatty acids (upper panels) and amino acids (lower panels) in the ciliates *B. planctonicum* and *U. farcta* fed the algae *C. phaseolus*, and the heterotrophic protists *Cyclidium sp.* and *C. paramecium* fed the "bacteria + rice" diet. Abbreviations are as follows: SAFA=saturated fatty acids, MUFA=monounsaturated fatty acids, PUFA=polyunsaturated fatty acids, EPA=Eicosapentaenioc acid, DHA=Docosahexaenoic acid; His=histidine, Thr=threonine, Arg=argenine, Trp=tryptophan, Met=metheonine, Val=valine, Phe=phenylalanine, Ile=isoleucine, Leu=leucine, Lys=lysine.

2.4 DISCUSSION

The majority of the dietary biochemical components were also observed in the heterotrophic protists. However, the protists ensured higher concentrations of several biochemical compounds than their algal diet, a pattern already observed for other herbivores (Raubenheimer, 1992). Moreover, differences in fatty acid and amino acid composition and concentrations among protist species suggest species-specific features in the metabolism of those biochemical compounds in the studied heterotrophic protists.

2.4.1 Fatty Acids

One could expect to find high fatty acid concentrations in heterotrophic protists fed a diet rich in fatty acids. If so, *Balanion* and *Urotricha* should contain higher fatty acid concentrations than *Cyclidium* and *Chilomonas*, as the algal diet had

higher concentrations of saturated and polyunsaturated fatty acids than the "bacteria + rice" diet. In fact we observed significant higher total SAFA, MUFA, and PUFA concentrations in the ciliates fed the algae than in the protists fed the "bacteria + rice" diet.

However, concentrations of monounsaturated fatty acids did not differ significantly between the algal and "bacteria + rice" diets. Since we found higher concentrations of monounsaturated fatty acids in both *Balanion* and *Urotricha* than in *Cyclidium* and *Chilomonas*, some metabolic differences in accumulation of monounsaturated fatty acids are supposed to occur among those protist species. These mechanisms could also be responsible for the high concentrations of some polyunsaturated fatty acids in the protists, despite low dietary concentrations (e.g. 16:2ω4, 18:3ω3, and the 22(ω6) fatty acids). Metabolic mechanisms underlying the accumulation of mono- and polyunsaturated fatty acids in the protists may include high fatty acid assimilation efficiencies and protist ability to preferentially use self-synthesized carbohydrates or other organic compounds as a source of energy. In both cases, carbon-specific concentrations of essential compounds obtained in the diet are expected to increase in the heterotrophic protists. In addition, the protist species we investigated may have compensated for occasionally low concentrations of mono and polyunsaturated fatty acids in their diet simply by exhibiting high ingestion rates. In this case, the cellular amount of fatty acids in the protists would be originated only from the diet, given that the studied protists are able to retain fatty acids, but not able to synthesise them. An estimate of the ingestion rates needed to reach the EPA concentrations we measured in the protists, versus known published ingestion rates for *Balanion planctonicum* (190 *Cryptomonas* cells ciliate^{-1} day^{-1}, Müller and Schlegel, 1999) and *Cyclidium* sp. (28000 bacteria ciliate^{-1} day^{-1}, Šimek et al., 1994) makes this a reasonable assumption. As we took samples for biochemical analysis around the sixth day of the growth phase, all protist species we investigated may have been able to obtain their entire cellular fatty acids from the diet. It muss be kept in mind, however, that estimates based on published ingestion rates have a very speculative character, although we have worked with the same species.

However, efficient ingestion rates alone cannot explain the higher carbon-specific concentrations of mono- and polyunsaturated fatty acids in the heterotrophic protists. For instance, despite the lower EPA cellular concentrations observed in *Cyclidium*, which obviously reflect its small size, this ciliate had carbon-specific EPA concentrations, which were similar to the EPA concentrations found in *Balanion* and *Urotricha*. Even if *Cyclidium* was able to get its entire cellular EPA amount from the diet, EPA incorporation efficiency in this species would need to

be considerably higher than in both *Balanion* and *Urotricha*, in order to result in similar carbon-specific concentrations. Moreover, since linolenic acid (18:3ω3), a precursor of EPA and DHA (see Appendix 1) was present in *Cyclidium* (Table 1) its conversion into EPA may be likely to occur in this ciliate.

The experiments of Erwin and Bloch (1963) and Lees and Korn (1966) illustrated the ability of *Tetrahymena pyriformis* to take up and incorporate, and/or convert and incorporate a considerable variety of short-chain and long-chain fatty acids, and by doing so, to modify its fatty acid composition. Moreover, this ciliate is known to synthesize unsaturated fatty acids by two distinct pathways from palmitic acid (Koroly and Connor, 1976). First evidences for the synthesis of highly unsaturated fatty acids, like EPA and DHA, and also sterols by the heterotrophic dinoflagellate *Oxyrrhis marina* (Klein Breteler et al., 1999) suggest reconsidering the assumption that heterotrophic protists are unable to synthesize complex lipid molecules. However, synthesis of unsaturated fatty acids may be inefficient in phagotrophic protists. Inefficient conversion ability was claimed for zooplankton consumers, which showed enhanced growth and reproduction in media supplemented with EPA or DHA (Brett and Müller-Navarra, 1997; DeMott and Müller-Navarra, 1997). Our study suggests synthesis of DHA by *Chilomonas*, possibly using 18:3ω3 as a precursor (Table 1). This could indicate a good prey quality of *Chilomonas*, as indeed observed for *Daphnia* (Sanders and Porter, 1990; Lair and Picard, 2000). One could argue that DHA in *Chilomonas* might have originated from ingested rice particles, assuming that this flagellate is able to ingest and to digest plant matter. However, the polished rice we used did not contain EPA and DHA (Boëchat unpublished data). Moreover, *Cyclidium* was grown in the same medium and did not contain any DHA, the same being true for the "bacteria + rice" diet. On the other hand, although present in *Cryptomonas*, DHA was not found in *Balanion* and *Urotricha*. Possibly, *Balanion* and *Urotricha* have low metabolic demands for DHA, or are unable to convert DHA from the polyunsaturated fatty acid 18:3ω3.

The SDA based on fatty acid data suggested a relative independence of our heterotrophic protists from dietary fatty acids. The polyunsaturated fatty acids were the best parameter in separating *Balanion* and *Urotricha* from their algal diet, the same being valid for *Cyclidium* and *Chilomonas*. All protists as well as their respective diets were discriminated already at the first and second roots, with clear differences originating from a broader palette of polyunsaturated fatty acids than observed for the SDA based on saturated or monounsaturated fatty acids. Although these fatty acid classes were also able to significantly discriminate among protist species and between diets, the composition of polyunsaturated fatty

acids seems to be more diverse and maybe strongly affected by species-specific metabolism. This is not surprisingly, since energetic costs of building polyunsaturated fatty acids are much higher than those for saturated or monounsaturated fatty acids. Also the presence of a broader number of accessory enzymatic systems is necessary to insert double bounds into the fatty acid carbon chain (Stryer, 1995). Heterotrophic protists fed the same diet were only separated at the third or fourth root of the SDA based on saturated or monounsaturated fatty acids, which indicates a rather similar and conservative composition among those species, which could arises partially from the diet or perhaps from similar features in metabolism and synthesis of those fatty acid classes. The SDA also suggested a more efficient accumulation of dietary saturated and monounsaturated fatty acids by *Cyclidium* and *Chilomonas*, as those species were clearly separated from their "bacteria + rice" diet at the first canonical root of the analyses. *Balanion* and *Urotricha* were still placed together with their algal diet at the first root of the SDA based on saturated fatty acids, which indicates a rather similar composition in those ciliates and in *Cryptomonas*.

We found high concentrations of polyunsaturated fatty acids in the "bacteria + rice" diet, in contrast to previous studies (Kaneda, 1991; Harvey et al., 1997). This might have resulted from an indirect influence of small rice particles from the culture medium, still present after filtering the cultures subsequently through 10 μm and 5 μm meshes (see sample preparation). Unfortunately, a quantification of that influence was not possible. Microscopic examinations, however, gave no evidence for high numbers of rice corn particles in our samples. Except for linoleic and linolenic acid, polished rice corns do not contain measurable amounts of long chain fatty acids (Souci et al., 1994). Fatty acid analyses of the rice corns used for our study confirmed this assumption (Boëchat, unpublished data), although we found high amounts of the saturated fatty acid 24:0 in our rice samples. Nevertheless, the results on dietary polyunsaturated fatty acids in the "bacteria + rice" samples should be interpreted with caution and should be considered when comparing our results with natural bacterial assemblages.

2.4.2 Amino Acids

Although the amino acid composition in the heterotrophic protists generally reflected that of their diet, carbon-specific concentrations were higher in the protists than in their diet. Moreover, carbon-specific amino acid concentrations differed species-specifically among the four investigated heterotrophic protists (Table 3). Again, metabolic capabilities of the protists involving ingestion and assimilation efficiencies may be the underlying mechanisms explaining the

discrepancies between protists and diet. Amino acid requirement and assimilation in *Tetrahymena* has been shown to be dependent on the concentrations of other nutrients in the culture medium such as sodium (Holz, 1973). This ciliate is also able to recover changes in cell volume resulting from changes in extracellular osmolarity, partially by adjusting the intracellular concentration of free amino acids (Dunham, 1973). Moreover, similar to plants and bacteria, which are able to synthesize their entire set of essential amino acids (Stryer, 1995) amino acid synthesis in heterotrophic protists may be expected to occur. Since different biosynthetic pathways for a single amino acid can differ among species (Umbarger, 1981), this may have contributed to the observed differences among protist species (e.g. the higher carbon-specific concentrations in *Chilomonas* than in *Cyclidium*).

The tendency of higher amino acid concentrations in the protists than in their diet, also observed for fatty acids, strengthens our hypothesis that heterotrophic protists can compensate for low dietary biochemical contents, simply by exhibiting high ingestion rates and high assimilation efficiencies. Here, dietary biochemical composition should have only a limited influence on the biochemical composition of the heterotrophic protists. The actually determining factors are possibly the metabolic features of the protists. This possibility is fascinating and deserves further investigation. Experiments with radioactively labeled amino acids are the next necessary step to clarify species-specific features in amino acid metabolism of heterotrophic protists.

2.4.3 Cell-Specific Concentrations of Biochemical Compounds

When considering overall energy uptake by predators, data on carbon-specific biochemical concentrations are important, because carbon provides a good biomass and energy estimate. However, in light of the entire feeding process, which includes capture of prey, handling time and ingestion of prey, the prey size (Mohr and Adrian, 2001) and consequently the cell-specific biochemical content is crucial. There is a trade-off between the effort of prey handling and the nutrition of the single prey. Cell-specific fatty acid concentrations in both *Balanion* and *Urotricha* significantly exceeded those found in *Cyclidium* and *Chilomonas* (Fig. 3). With respect to the fatty acid input per single predator/prey encounter, higher fatty acid concentrations in *Balanion* and *Urotricha* could guarantee a better food quality for rotifers fed algivores as compared to bacterivores, a fact already observed (Mohr and Adrian, 2002b). In contrast, amino acid concentrations seemed to be not so dependent of diet composition and protist cell size. Thus, amino acid concentration cannot be regarded as a good indicator of protist

nutritional quality when comparing algivorous and bacterivorous protist prey. This is consistent with findings of rather constant amino acid concentrations in freshwater microalgae (Ahlgren et al., 1992) and rotifers (Frolov et al., 1991), and is supported by previous studies, which demonstrate the absence of relationship between prey amino acid composition and nutritional quality (Watanabe et al., 1978; Frolov et al., 1991).

2.4.4 Trophic Transfer in Food Webs

In aquatic ecosystems, the second level of the Eltonian biomass pyramid can be larger than the producers' level (Brett and Müller-Navarra, 1997). In our case, the protist matter was more concentrated in various chemical compounds than the algal or "bacteria + rice" matter. Therefore, heterotrophic protists might be able to ingest and/or accumulate these compounds efficiently. Although we did not measure ingestion and assimilation rates, we assume that both *Balanion* and *Urotricha*, and especially *Cyclidium* were probably better in accumulating monounsaturated and some polyunsaturated fatty acids (Fig. 1). All protist species, but especially *Chilomonas*, seemed to be efficient in amino acid accumulation. Overall, with respect to various essential chemical compounds, heterotrophic protists may be viewed as prey of upgraded quality at an early stage in aquatic food webs. The fact that the biochemical (the present study) and elemental compositions (Caron et al., 1990) of heterotrophic protists can indeed differ from dietary composition suggest species-specific differences in metabolic pathways of heterotrophic protists. This possibility may stimulate further research concerning assimilation and metabolism of organic matter in heterotrophic protists. In light of trophic interactions and trophic transfer efficiency, our results may be very useful to substantiate the view of heterotrophic protists as an important link in transferring biochemical matter and energy between the microbial and the classical food web.

3. STEROL COMPOSITION OF FOUR FRESHWATER HETEROTROPHIC PROTISTS AN THEIR DIET

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(*Journal of Chemical Ecology*, under revision)

Keywords: Heterotrophic protists; Sterol composition; Sterol metabolism; Triterpenoids; Consumer-diet interactions

ABSTRACT

Heterotrophic protists represent an important link in aquatic food webs as they transfer energy and biochemical matter from their bacterial and algal prey to mesozooplankton predators. Our aim was to understand how the sterol composition of the diet affects the sterol composition of four freshwater heterotrophic protists. We analyzed the sterol composition of two algivorous protists (*Balanion planctonicum* and *Urotricha farcta*) and two bacterivorous protists (*Cyclidium sp*. and *Chilomonas paramecium*) as well as of their diet – the cryptomonad *Cryptomonas phaseolus* for *B. planctonicum* and *U. farcta*, and a mixture of bacteria growing on two different rice types for *Cyclidium sp*. and *C. paramecium*. The sterol composition of the protists did not generally resemble that of their diet, since some discrepancies were observed. Ergosterol was the main sterol in *C. phaseolus*, whereas stigmasterol was dominant in both *B. planctonicum* and *U. farcta*. The diets consisting of "bacterial + rice" were both rich in cholesterol and sitosterol, whereas cholesterol and stigmasterol were the major sterols in *Cyclidium sp.* and *C. paramecium* respectively. The occasionally higher sterol concentrations in the protists than in their diet suggest high sterol accumulation efficiencies by the protists. Moreover, ergosterol synthesis is likely to occur in *C. paramecium*. We conclude that the dietary sterol composition influences the sterol composition of the four freshwater heterotrophic protists; however, species-specific differences in sterol metabolism ultimately determine the sterol composition of the protists.

3.1 INTRODUCTION

Generally, the biochemical composition of a consumer is expected to resemble that of its diet. This was already shown for different mesozooplankton predators in food quality studies involving fatty acid (e.g. Harvey et al., 1997) and amino acid composition (e.g. Guisande et al., 2000). If this is also the case for heterotrophic protists, the biochemical composition of their diet will affect or even determine the biochemical composition of the protists. Consequently it may affect protist nutritional quality for zooplankton predators if we consider the potential limiting role of some biochemical compounds for zooplankton (DeMott and Müller-Navarra, 1997; Becker and Boersma, 2003; Ravet et al., 2003). If this is not the case, discrepancies in the biochemical composition between heterotrophic protists and their diet may result from differences in prey capture, ingestion, and digestion rates as well as in the capability of heterotrophic protists to synthesize biochemical compounds. Indeed, initial investigations of the biochemical composition of four freshwater heterotrophic protists showed that the fatty acid and amino acid composition of the protist species generally resembled that of their diet. Nevertheless, some polyunsaturated fatty acids were observed in the protists, which were not present in their diet (Boëchat and Adrian, submitted). With regard to nutritional quality, those heterotrophic protists can be considered as prey of upgraded quality for their zooplankton predators, since the polyunsaturated fatty acids referred to are known to be essential for many zooplankton predators (Brett and Müller-Navarra, 1997; Ravet et al., 2003).

Along with essential polyunsaturated fatty acids and amino acids, sterols have recently gained much attention as biochemical components which confer food quality of planktonic prey (Von Elert, 2002; Hassett, 2004; Martin-Creuzburg and Von Elert, 2004). Sterols share, along with phospholipids, a structural function in membranes, but in terms of polarity they are grouped with triacylglycerols in the neutral lipids (Parrish, 1999). In addition to controlling membrane fluidity and permeability, sterols also form sexual hormones, sterol alkaloids, and act as vitamins. In some higher plants, sterols have specific functions in cell proliferation, signal transduction, and also as modulators of the activity of membrane-bound enzymes (Volkman, 2003). Additionally, sterols have also been used as markers in marine and freshwater sediments, since some of them are typical for certain taxonomic groups (Harvey and McManus, 1991; Elhmmali et al., 2000; Hudson et al., 2001). While cholesterol is the predominant sterol in animal cells, plant membranes are rich in several types of ‘phytosterols’, which are similar in structure to cholesterol but include a methyl or ethyl group at

carbon-atom 24. Additionally, phytosterols are also thought to stabilize plant membranes (Moreau et al., 2002).

Much of the information concerning sterols in plankton organisms relates to the sterol composition of the phytoplankton (see Patterson, 1991 for a review). Algal sterol composition is generally similar to that observed in higher plants, but algal classes differ in their predominant sterols. By far the widest range of sterols has been found in *Chlorophyceae* (Patterson, 1991) including (22*E*)-ergosta-5,7,22-trien-3β-ol (ergosterol), cholest-5-en-3β-ol (cholesterol), (22*E*)-ergosta-5,22-dien-3β-ol (brassicasterol), [24(24')*E*]-stigmasta-5,24(24')-dien-3β-ol (fucosterol), and many others (Volkman et al., 1994). Although the occurrence of sterols in *Cyanophyceae* is controversial (Volkmann, 2003), cholesterol, 24α-ethylcholest-5-en-3β-ol (sitosterol), and brassicasterol were found in this group (Volkman, 1986), while (22*E*)-(24*S*)-24-methylcholesta-5,22-dien-3β-ol (epibrassicasterol) was the major sterol found in *Cryptophyceae* (Goad et al., 1983; Gladu et al., 1990). Nearly all dinoflagellates contain cholesterol and 4,23,24-trimethylcholest-22-trien-3β-ol (dinosterol) as the most common sterols (Mansour et al., 1999).

In sharp contrast to the vast literature on algal sterols, the sterol composition of bacteria and heterotrophic protists has been only marginally been considered. Except for some methanotrophic species (Schouten et al., 2000), bacteria are generally considered not to produce sterols, or only in non-significant amounts (but see Kohl et al., 1983; Sorkhoh et al., 1990). Despite studies on marine species (Harvey and McManus, 1991; Ederington et al., 1995; Harvey et al., 1997; Breteler et al., 1999; Sul et al., 2000), the sterol composition of freshwater heterotrophic protists is still a 'black box'. The only exceptions are studies on freshwater flagellates of the genus *Chilomonas*, which contained 24α-ethylcholesta-5,22(*E*)-dien-3β-ol (stigmasterol) as the predominant sterol (Patterson, 1991), and the freshwater bacterivorous ciliate *Tetrahymena pyiriformis*, for which a triterpenoid alcohol – tetrahymanol (gammaceran-3β-ol) – was first isolated and described as its major neutral lipid (Mallory et al., 1963). This alcohol as well as hopanoid terpenes has also been found in marine ciliates (Harvey and McManus, 1991; Harvey et al., 1997), and may exhibit equivalent regulatory functions as those attributed to sterols (Ferguson et al., 1975; Ourisson et al., 1987).

To determine whether the sterol composition of freshwater heterotrophic protists resembles the dietary sterol composition, we analyzed the sterols of four protist species (the ciliates *Balanion planctonicum*, *Urotricha farcta*, *Cyclidium sp*., and the flagellate *Chilomonas paramecium*) as well as the sterols of their diet (the

cryptomonad *Cryptomonas* phaseolus, which served as prey for *B. planctonicum* and *U. farcta*, and two different bacterial assemblages growing on different rice types – unpolished rice for *Cyclidium* sp. and polished rice for *C. paramecium*). We found discrepancies between protists and diet in both the composition and the relative and absolute concentrations of sterols. We discuss these discrepancies in light of possible metabolic mechanisms already described for other protists, including biosynthetic pathways and sterol conversion mechanisms. The present study suggests that heterotrophic protists are able to efficiently accumulate, convert, or even synthesize some sterols, especially phytosterols. Thus, our results support the hypothesis that freshwater heterotrophic protists are organisms of upgraded food quality, with respect to the essentiality of some sterols for zooplankton predators.

3.2 METHODOLOGY

3.2.1 Cultures

The ciliates *Balanion planctonicum* (average biovolume 3256 ± 1331 μm^3) and *Urotricha farcta* (2778 ± 1707 μm^3) were cultured in WC medium (Guillard and Lorenzen, 1972) in weekly diluted batch cultures incubated at 17°C under a 12:12 h light : dark regime. The ciliates were fed the cryptomonad *Cryptomonas phaseolus* (392 ± 125 μm^3), obtained from the Algal Collection of the University of Göttingen, Germany, and cultured in WC medium at 17 ± 1°C under a 16:8 h light : dark regime. The feeding spectrum of both ciliates includes bacteria, small algae (*Cryptomonas*, *Ochromonas*), and organic debris (optimal particle size 8 – 12 μm; Müller, 1991; Foissner et al., 1999). Nevertheless, our ciliate cultures were only able to efficiently grow when fed on *C. phaseolus*, thus providing sufficient biomasses for biochemical analysis.

The flagellate *Chilomonas paramecium* (403 ± 288 μm^3) and the bacterivorous ciliate *Cyclidium* sp. (1315 ± 617 μm^3) were cultured in Volvic water (a spring water, poor in minerals, sold worldwide by Société des Eaux de Volvic, Puy- de-Dôme, France) at 18 ± 1°C under a 16:8 h light : dark regime. *Chilomonas* was fed on a mix of bacteria grown on polished rice (referred to as "bacteria + polished rice" diet, since the protists might have been able to ingest rice particles along with bacteria in the medium) and *Cyclidium* was fed a mix of bacteria grown on unpolished rice (referred to as "bacteria + unpolished rice", for the same

reason). Bacteria and small rice particles could not be completely separated (see sample preparation and extraction). The reason why we used two different bacterial assemblages was because *Cyclidium* was only successfully cultured on bacteria growing on unpolished rice[1], the same being the case for *Chilomonas* growing on polished rice. Attempts to cultivate both species on bacteria growing on the other respective type of rice as well as on wheat corns were not successful. The ciliate cultures were able to grow for a couple days before collapsing, but never yielded biomasses sufficiently high for biochemical analysis. Sterol analyses of dried rice samples of both types revealed significant differences (tested using the Mann-Whitney *U*-test, Statistica for Windows, version 5.01, Stat Soft). Thus, an ultimate comparison of the sterol composition between our *Cyclidium* and *Chilomonas* cultures is not possible. However, the fact that the two protist species were fed bacterial assemblages growing on rice of different biochemical composition provides additional information regarding species-specific differences on sterol metabolism and synthesis in these species.

To investigate if the biochemical composition of heterotrophic protists resembles that of their diet, one could either (1) compare different species to their respective diets or (2) compare several cultures of one protist species raised on different diets. We applied the first approach because our species had very specific diet requirements and did not grow on alternative diets. Further, our study aimed at acquiring basic information about the biochemistry of a broader number of stable protist cultures, to facilitate future research on the nutritional quality of these protists for zooplankton predators.

Although our results suggest a series of possible mechanisms involved in the biochemical metabolism of the different protist species, a firm conclusion about the extent in which dietary composition determines biochemical composition of heterotrophic protists is hardly possible. For doing that, the next step should be to investigate the biochemical composition of single protist species raised on different diets or grown under different trophic modes.

3.2.2 Sample Preparation and Extraction

Sterol analyses were performed for heterotrophic protist species and their diet, and are given as percentage on the total sterol concentration (referred to as relative concentrations) and as carbon-specific concentrations (referred to as absolute concentrations). As organisms may show similar relative sterol concentrations,

[1] The *Cyclidium* culture analysed in the present study is not the same considered in the chapter 3. This new culture, obtained from Prof. K.O. Rothhaupt, was not able to grow efficiently on polished rice corns.

despite different absolute concentrations, both concentration forms are considered when comparing protists and their diet. Additionally, the sterol composition of both polished and unpolished dried rice corns was analyzed and the results are given as µg per milligram dry weight.

For biomass estimates, samples of ciliates, flagellates, and the algal diet were fixed with Lugol's solution and counted under a stereomicroscope at x20 magnification. Bacteria samples (1 mL) were taken from all protist cultures as well as from the algal and "bacteria + rice" diets. Samples were then fixed in 2% formaldehyde, filtered onto 0.2 µm pore size black polycarbonate membranes, stained with 4,6-diamidino-2-phenylindol (DAPI), and counted with an epifluorescent microscope (excitation wavelength 450 – 490nm combined with an FT 510 beam splitter and a LP 520 suppression filter) (Porter and Feig, 1980). Carbon concentrations in heterotrophic protists and their diet were derived from cell volume estimates using carbon : biovolume conversion factors of 0.10 pgC μm^{-3} for the alga *Cryptomonas* (Montagnes, 1994), 0.19 pgC μm^{-3} for heterotrophic protists (Putt and Stoecker, 1989), and 0.125 pgC μm^{-3} for the bacteria (Pelegri et al., 1999).

Due to their similar cell dimensions, *Balanion* and *Urotricha* could not be completely separated from their algal food via filtration and/or centrifugation. *Cryptomonas* biomass accounted for 4–16% of the carbon biomass in the protist samples for analyses. To a lesser extent, this was also the case for the protist cultures growing on bacteria and rice corns, although they were previously filtered successively over a 10 µm and a 5 µm mesh. Due to this previous filtration, bacterial biomass was reduced to less than 20% of the total carbon content in the cultures. Nevertheless, rice particles and bacterial cell smaller than 5 µm were still present in the samples for sterol analyses.

Since a complete separation of heterotrophic protists and their diet was not possible, we adopted a subtraction method, in order to accurately estimate the sterol composition and concentration of the protists. By knowing the mass-specific sterol contents of the diet, obtained from repeated analysis of either the algal or bacteria + rice cultures, we determined the sterol concentration of the protists indirectly through subtraction. By reducing dietary biomass to less than 20% of the total biomass in the protist samples for analysis, dietary sterol concentrations in these samples corresponded to less than 3% of the total concentration measured for the sample. Hence, subtracting dietary biomass concentrations from the overall concentration measured in the incompletely separated "protist + diet" samples minimized small biases due to incomplete

separation. Further, the accuracy of the correction was guaranteed by analyzing a large number of samples (at least 2–3 samples per culture, for a minimum of four cultures per protist species).

For sterol analyses, 300 to 700 mL of each culture of heterotrophic protists and the algal diet were filtered in 2–3 replicates on pre-incinerated (550°C, 4 h) GF/C Whatman glass fiber filters. Samples of bacterial cultures were collected on GF/F Whatman glass fiber filters. The analyses were performed for at least four different batches of each protist-diet combination and the individual dietary cultures. We collected protist cells at the end of the exponential growth phase because algal and bacterial densities were minimal in the cultures by this time (see sample preparation). Nevertheless, protist cells were still within the exponential growth phase. During this growth phase the food quality of green algae (*Scenedesmus*) had been found to be the highest for *Daphnia* (Sterner, 1993). For the sterol analyses of both rice types in dried form, 10 mg of homogenized rice sample were analyzed in triplicate. Rice samples and filters containing the organisms were immediately extracted with chloroform-methanol 2:1, v/v (Folch et al., 1957) and homogenized by sonication for 10 min at 5000 cycles per min. (Ultrashall-Desintegrator USD 20, VDE Wiss. Gerätebau, Berlin). After sonication, an internal standard was added to the samples (5α-cholestane, 0.2 mg mL^{-1}). The samples were then allowed to extract for 3 h at 20°C. After extraction, samples were dried under nitrogen flux and promptly stored at –20°C until analysis.

3.2.3 Sterol Analyses

Extracts were subjected to alkaline hydrolysis (saponification) by the addition of 5 mL 1 N potassium hydroxide (KOH) solution in 80% methanol (MeOH), followed by heating at 80°C for 30 min. After cooling, 10 mL distilled water and 5 mL n-hexane were added to the samples (Gordon and Collins, 1982). The n-hexane phase containing the free neutral lipids were separated and subsequently silylated by adding 25 µL Bis (trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane (BSTFA), 75 µL pyridine, and heating the sample at 60°C for 30 min (Breteler et al., 1999). Sterol silylether derivatives were analyzed using a gas chromatograph (Agilent 6890) equipped with a mass selective detector (Agilent 5973-N) and a fused silica capillary column (HP-5MS; 60 m x 0.32 mm x 0.25 µm). The carrier gas (helium) was held constant at 1.3 mL min^{-1}. The temperature of the PTV (programmed temperature vaporization) inlet – operating in splitless mode – was 300°C (initial temperature 100°C, 720°C min^{-1}). The temperature of the detector interface was 280°C. The following temperature

program was employed: 150°C initial temperature for 3 min, than heating at 4°C min^{-1} up to 300°C, and maintained for 40 min. Sterol silylether derivatives were identified by their retention times and their mass spectra in full scan mode (SCAN) previously calibrated with individual sterol standards (Sigma-Aldrich). The mass spectra of the detected sterols were compared with the mass spectra of our self-generated spectra library stored in a dedicated data system (Agilent Chemstation). Sterol silylether derivatives were quantified by selective ion monitoring (SIM) at the two most intensive ions at the molecular ion cluster. Calibration curves ranged between 0.04 and 0.4 μg sterol per mL injected sample. Sterols are provided as percentages of the total sterol pool (relative amounts) and as absolute concentration per carbon biomass, whenever a commercial standard was available. We refer to the sterols by their trivial names throughout the text and in the tables, in order to facilitate reading and comparisons. However, we indicated the classic nomenclature when first referencing to the sterol common name (there are currently two main nomenclatures following the IUPAC-IUB recommendations; see Moreau et al. [2002] for a list of synonyms currently used). Although not quantified in terms of absolute concentrations, some additional neutral lipids were identified by their mass spectra and are provided as percentages of the neutral lipid fraction.

3.2.4 Statistical Analyses

Considering the non-normality of data distribution, differences in sterol absolute concentrations and relative amounts (percentages) between the heterotrophic protists and their diet were tested with the Mann-Whitney *U*-test (Statistica for Windows, version 5.01, Stat Soft). Differences in the sterol composition between polished and unpolished rice samples were tested with the Mann-Whitney *U*-test as well.

3.3 RESULTS

In contrary to our hypothesis, the sterol composition of the heterotrophic protists did not generally resemble that of their diet (Tables 5 and 6). Discrepancies in sterol occurrence and concentration between heterotrophic protists and their diet were observed for *Balanion* and *Urotricha*, and especially for *Chilomonas* and *Cyclidium*. Considering relative concentrations, ergosterol was the predominant sterol in the algal diet *Cryptomonas* (42.8%), followed by brassicasterol (15.9%),

cholesta-5,24-dien-3β-ol (desmosterol, 12.4%), stigmasterol (9.8%), cholesterol (6.5%), and 24α-ethylcholestan-3β-ol (stigmastanol, 5.1%) (Table 5). Stigmasterol was the predominant sterol in *Balanion* (49.3%), along with brassicasterol (20.6%), desmosterol (18.4%), (24R)-24-methylcholest-5-en-3β-ol (campesterol, 5.2%), and cholesterol (1.9%). Interestingly, ergosterol was not detected in *Balanion* as well as 3β-hidroxy-5α-cholestane (dihydrocholesterol). *Balanion* contained higher relative amounts of stigmasterol, campesterol, desmosterol, 24-stigmasta-5,7,24(28)-trien-3β-ol, and of another non-identified sterol than its algal diet *Cryptomonas* (Mann Whitney *U*-test, $P<0.05$; Table 5). Stigmasterol was also the predominant sterol in *Urotricha* (26.7%), followed by ergosterol (20.9%), brassicasterol (13.9%), desmosterol (12.2%), sitosterol (9.0%), and cholesterol (7.5%) (Table 5). *Urotricha* presented higher relative amounts of stigmasterol, sitosterol, 24-stigmasta-5,7,24(28)-trien-3β-ol, and of the non-identified sterol than its algal diet *Cryptomonas* ($P<0.05$; Table 5).

The diet consisting of "bacteria + polished rice" displayed a smaller spectrum of sterols than the diet consisting of "bacteria + unpolished rice" (Table 6). Cholesterol was the predominant sterol (65.8%) identified in "bacteria + polished rice" samples (*Chilomonas*' diet), followed by low relative concentrations of dihydrocholesterol (13.5%), stigmastanol (10.9%), and stigmasterol (6.1%). Sitosterol was the predominant sterol (49.9%) found in "bacteria + unpolished rice" samples (*Cyclidium*'s diet), followed by cholesterol (17.3%), campesterol (12.7%), stigmasterol (12.2%), and stigmastanol (6.4%). Other sterols found in low relative concentrations were 5α-cholest-7-en-3β-ol (lathosterol, 0.5%), desmosterol (0.5%), dihydrocholesterol (0.3%), and ergosterol (0.3%). Unlike the sterol composition found in "bacteria + unpolished rice", campesterol, sitosterol, and ergosterol were not detected in the samples of "bacteria + polished rice" (Table 6). Differences in the sterol composition between both "bacteria + rice" samples basically stemmed from the differences found for the two types of rice (Fig. 1). This should be considered when comparing our results with natural bacterial assemblages. The significantly higher concentrations of campesterol, stigmasterol, sitosterol, and stigmastanol in unpolished rice ($P<0.05$; Fig. 1) were probably due to sterols attached to the shell of this rice type. All other sterols were detected in both rice types in similar concentrations, while ergosterol and brassicasterol were not detected in any rice type. Due to the discrepancies in the sterol composition of the two "bacteria +rice" assemblages, a direct comparison of the two protist species fed "bacteria + rice" was not possible (i.e. *Cyclidium* and *Chilomonas*).

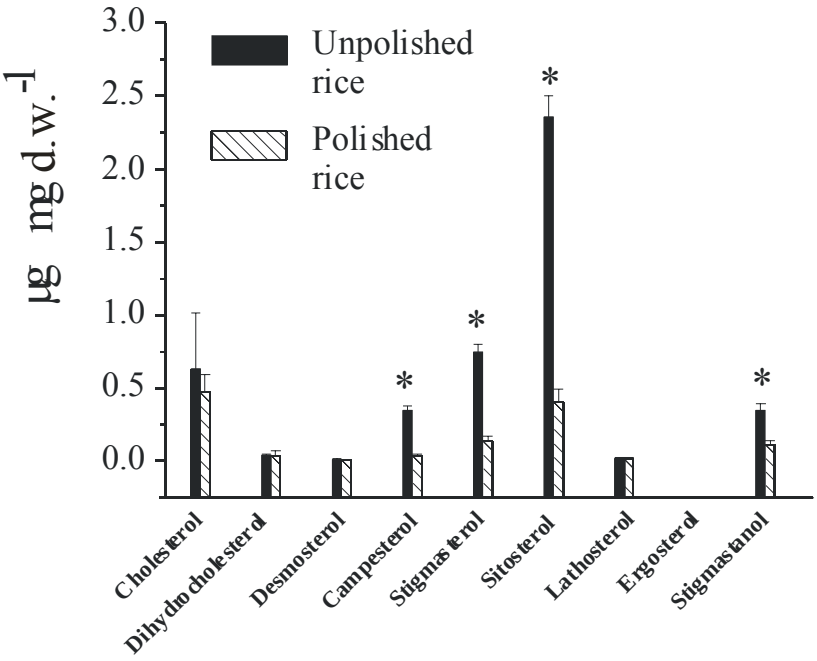

Fig. 1 – Absolute sterol concentrations (mean ± S.D.) in polished versus unpolished rice used as food sources for the bacterial diet in the cultures of *Cyclidium sp*. and *Chilomonas paramecium*. Asterisks represent significant differences at 95% confidence ($P<0.05$, Mann-Whitney *U*-test).

In contrast to the sterol composition observed in its "bacteria + polished rice" diet, *Chilomonas* contained stigmasterol (39.7%) and sitosterol (32.1%) as the main sterols, followed by campesterol, ergosterol, cholesterol, desmosterol, and lathosterol (Table 6). Interestingly, campesterol, sitosterol, and ergosterol were not found in the diet of *Chilomonas*. On the other hand, although dominant in its diet, cholesterol, and stigmastanol were of minor importance in *Chilomonas* ($P<0.05$; Table 6). *Chilomonas* did not contain dihydrocholesterol, although this sterol was observed in the "bacteria + polished rice" diet. Relative amounts of stigmasterol were higher in *Chilomonas* than in its diet consisting of "bacteria + polished rice" ($P<0.05$; Table 6). Moreover, *Chilomonas* contained brassicasterol and the 24-stigmasta-5,7,24(28)-trien-3β-ol, which were not observed in the "bacteria + polished rice" diet. *Cyclidium* fed "bacteria + unpolished rice" produced a different sterol composition than that observed for *Chilomonas*, which was fed "bacteria + polished rice" (Table 6). Cholesterol was the major sterol found in *Cyclidium* (70.6%) followed by sitosterol, stigmasterol, and dihydrocholesterol. Nevertheless, all sterols detected in *Cyclidium* were also observed in its diet, although not necessarily in the same proportions (Table 6). For example, cholesterol was the second dominant sterol in the diet (17.3%), but

represented the major fraction of the sterol pool in *Cyclidium* (70.6%). Interestingly, dihydrocholesterol was found in the algal and in both "bacteria + rice" diets, but it was not detected in the heterotrophic protists, except for *Cyclidium*.

When considering the absolute sterol concentration – per carbon units – in the heterotrophic protists and their diet, some discrepancies were observed (Tables 5 and 6, values in parentheses). The concentrations of campesterol and stigmasterol were much higher in *Balanion* than in the algal diet, while stigmasterol and sitosterol reached higher concentrations in *Urotricha* than in *Cryptomonas* ($P<0.05$). *Cryptomonas* had higher concentrations of desmosterol and ergosterol than *Urotricha* ($P < 0.05$). However, overall sterol concentrations in *Cryptomonas* (26.8±21.2 μg mgC^{-1}), *Balanion* (27.6 ± 2.2 μg mgC^{-1}), and *Urotricha* (20.6 ± 12.1 μg mgC^{-1}) did not differ significantly ($P>0.05$).

Discrepancies between protist and dietary absolute sterol concentrations were especially evident for *Cyclidium* and *Chilomonas*. *Chilomonas* had higher concentrations of all sterols than its diet ("bacteria + polished rice") except for cholesterol, desmosterol, and stigmastanol ($P<0.05$; Table 6). The opposite was found for *Cyclidium*, which had lower concentrations of all sterols compared with those in its diet ("bacteria + unpolished rice"), with the exception of the 27-carbon sterols cholesterol, dihydrocholesterol, desmosterol, and lathosterol ($P<0.05$; Table 6). The overall sterol concentration in *Chilomonas* (46.1 ± 19.1 μg mgC^{-1}) was higher than in its "bacteria + polished rice" diet (16.5 ± 8.3 μg mgC^{-1}), while the overall sterol concentration in *Cyclidium* (37.7 ± 20.7 μg mgC^{-1}) was much lower than in its "bacteria + unpolished rice" diet (90.4 ± 5.6 μg mgC^{-1}).

Two sterol precursors, the alcohol terpenoid tetrahymanol, and two tertepenoids of the hopane class (diplopterol and diploptene) were identified by their mass spectrometric patterns. Squalene, the sterol precursor in all biosynthetic pathways (Fig. 2), was detected in similar concentrations in all heterotrophic protists and in their diets (Tables 5 and 6). Cycloartenol was identified in *Cryptomonas*, *Urotricha*, *Chilomonas*, and *Cyclidium*. Cycloartenol is an intermediate precursor in the biosynthesis of phytosterols (Moreau et al., 2002). The alcohol tertepenoid Tetrahymanol, the hopanoids hopan-22-ol (diplopterol), and hop-22(29)-ene (diploptene) were detected in *Cyclidium* (11.5 %, 30.2 %, and 2.5 % of the total neutral lipids fraction). Trace amounts of hopan-22-ol were found in *Cryptomonas* and *Urotricha* (1.1 % of the total neutral lipids).

3.4 DISCUSSION

Along with certain essential fatty acids, amino acids, and other biochemical compounds, sterols can be viewed as nutritional quality indicators, since some of them are not synthesized *de novo* in animal tissues, and thus must be obtained from the diet. Consequently, the diet of a consumer is likely to determine its sterol composition, unless the consumer is able to modify dietary sterols and/or is able to synthesize sterols, given the presence of intermediate precursors. Here, we show that the dietary sterol composition influences the sterol composition of heterotrophic protists, since most sterols found in the diet were also found in the protists. For instance, high relative and absolute desmosterol concentrations were found in both *Balanion* and *Urotricha* as well as in their algal diet *Cryptomonas*. Stigmasterol was present in high relative and absolute concentrations in both *Cyclidium* and *Chilomonas* as well as in their diet consisting of "bacteria + rice". However, the predominant sterols in the protists – based on relative concentrations – were not necessarily the same as those observed in their diet. For example, stigmasterol comprised about 39 and 61% of the overall sterol composition in *Urotricha* and *Balanion*, respectively, but only 9.8% of the total sterols found in the algal diet *Cryptomonas*. Moreover, except for *Cyclidium* absolute sterol concentrations were generally higher in the protists than in their respective diets (Tables 5 and 6). Finally, the occurrence of some sterols in the heterotrophic protists (e.g. campesterol, ergosterol, sitosterol, brassicasterol, and 24-stigmasta-5,7,24(28)-trien-3β-ol in *Chilomonas*) along with the absence of others (e.g. ergosterol in *Balanion*) characterizes disparities from the expected resemblance between consumer and dietary composition. These discrepancies in both sterol composition and concentration between heterotrophic protists and their diet may stem from species-specific differences in mechanisms which are involved in sterol metabolism, like sterol accumulation, assimilation, and synthesis. Evidence for such differences concerning fatty acid and amino acid metabolism have already been found for the same protist species (chapter 1).

In the following we singled out the sterols whose occurrence and concentration between heterotrophic protists and their diet were highly different. We then discuss these discrepancies in light of possible metabolic and functional mechanisms based on evidence provided by our results along with mechanisms already described for other protist species.

The elevated absolute and relative stigmasterol concentrations especially in *Balanion*, *Urotricha*, and *Chilomonas* may have reflected the age of our cultures. In plants, the molar ratio of stigmasterol to other phytosterols was shown to increase during senescence (Stalleart and Geuns, 1994). By analyzing protist cultures originating from the end of the exponential growth phase, we may have included cells entering a "senescence" stage. On the other hand, the high relative and absolute stigmasterol concentration in the heterotrophic protists, despite low stigmasterol occurrence in their diets, makes us to ponder whether our protists are efficient in ingesting, assimilating, and/or synthesizing stigmasterol. High stigmasterol amounts in the protists suggest the importance of this phytosterol for growth within heterotrophic protists, as already demonstrated for the ciliate *Trimyema compressum* (Holler et al., 1993). Previous studies suggested that stigmasterol undertakes cholesterol analogous function in controlling permeability of cellular membranes (Piironen et al., 2000). Further efforts will be necessary to verify the role of stigmasterol in protist cells.

Ergosterol has been found to be the major sterol of several flagellates (Nes and McKean, 1977), amoebas (Raederstorff and Rohmer, 1987a,b), and some parasitic protozoa (Dixon et al., 1972; Furlong, 1989). Ergosterol, followed by brassicasterol, was the predominant sterol in our *Cryptomonas* cultures. Similar findings of brassicasterol and ergosterol as major sterols in other cryptomonads have already been reported (Goad et al., 1983). Ergosterol was the second major sterol found in our *Urotricha*. *Cryptomonas* can probably synthesize ergosterol, whereas *Urotricha* more probably obtained its ergosterol from *Cryptomonas*. However, ergosterol synthesis in *Urotricha* should not be excluded, since we detected the sterol precursor "cycloartenol" in *Urotricha*. *De novo* synthesis of sterols has been described for some amoebae species belonging to the genera *Acanthamoeba* and *Naegleria* (Raederstorff and Rohmer, 1987a,b). In those species, the $\Delta^{5,7}$ sterol ergosterol was the predominant sterol, with cycloartenol serving as a precursor (the numbers after the symbol Δ represent the position of the double bonds in the ring A of the sterol molecule).

In all sterol biosynthetic pathways, squalene is the 30-carbon precursor (Fig. 2), being a derivate from either mevalonate (the classic Bloch-Lynen mevalonate or MVA pathway) or pyruvate and glyceraldehyde (recently elucidated methylerythritol-phosphate or MEP pathway; Rohmer et al., 1996). Hence, we expected to find squalene in all organisms we analyzed, as actually observed. In animals, fungi, and dinoflagellates, squalene is converted to lanosterol, which is the precursor of most 27-carbon sterols, such as desmosterol and cholesterol (Volkman, 2003) (Fig. 2).

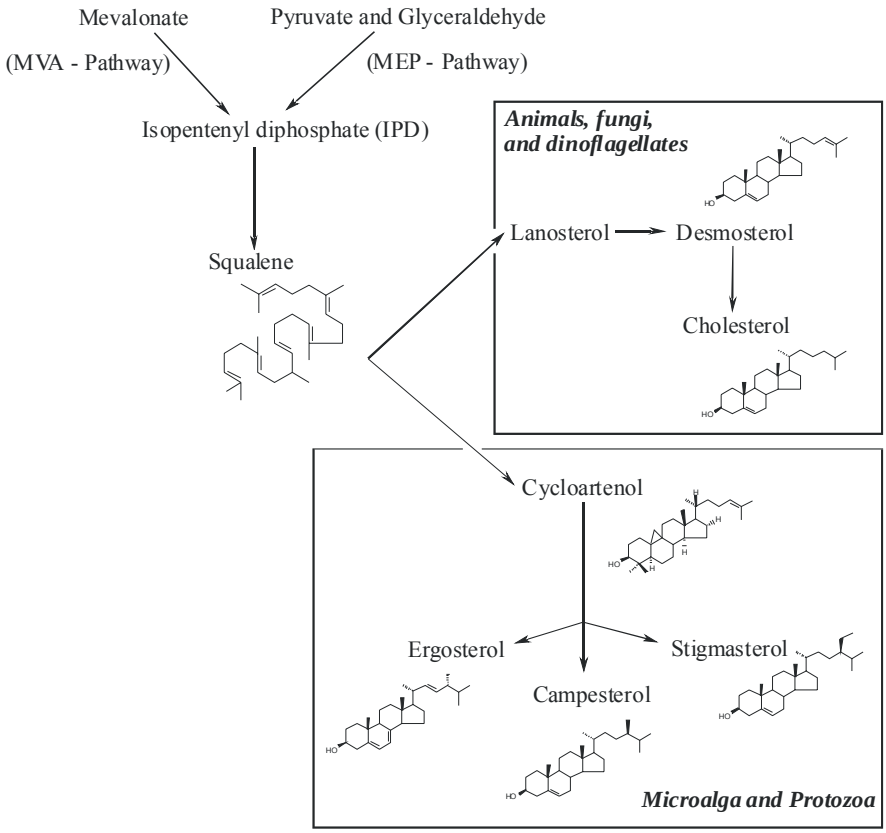

Fig. 2 – Sterol biosynthetic pathways. The Mevalonate pathway (MVA-pathway) and the recently elucidated Methylerythritol-Phosphate pathway (MEP-pathway) culminate in the synthesis of squalene. In animals, fungi, and dinoflagellates, squalene is converted to lanosterol, which is the precursor of desmosterol and cholesterol. In higher plants, most microalgae, and many heterotrophic protists squalene is converted to cycloartenol, which in turn is converted to phytosterols, like campesterol and ergosterol (see text for references).

In higher plants, most microalgae, and in many heterotrophic protists squalene is converted to the intermediate dimethylsterol cycloartenol through cyclization steps (e.g. Giner et al., 1991) (Fig. 2). Cycloartenol is then converted to cycloartanol, which is later converted to 28- and 29-carbon desmethyl phytosterols, such as campesterol and ergosterol. The presence of cycloartenol in our *Cryptomonas* and *Urotricha* samples may suggest ergosterol synthesis through conversion of cycloartenol in these species. Interestingly, ergosterol was not detected in *Balanion*, which probably metabolizes this sterol into another Δ^5 sterol, according to the conventional sterol biosynthetic sequence $\Delta^7 \rightarrow \Delta^{5,7} \rightarrow \Delta^5$ (Goad, 1981).

Campesterol, sitosterol, ergosterol, and brassicasterol were detected in *Chilomonas*, despite their absence in its "bacteria + polished rice" diet. Direct

assimilation of campesterol and sitosterol through ingestion of rice particles from the medium could explain the presence of these sterols in *Chilomonas*, since they were detected in the analyses of dried polished rice. The reason why those sterols were detected in the samples of dried polished rice but not in the "bacteria + polished rice" samples (medium samples) is unclear. Maybe the concentration of those sterols in the "bacteria + polished rice" samples were below the detection limits of our method. Although we could not quantify it, the biomass of rice particles present in the "bacteria + polished rice" samples might have been very low, as the cultures were successively filtered over 10 and 5 μm prior to the analyses. However, the dried polished rice and the "bacteria + polished rice" samples did not contain ergosterol and brassicasterol. The conversion of a Δ^5 sterol, such as cholesterol into ergosterol in *Chilomonas* could explain this discrepancy. The conversion of Δ^5 to $\Delta^{5,7}$ sterols is already known to take place in some heterotrophic protists (Goad, 1981) and *de novo* synthesis of ergosterol has recently been demonstrated to occur via incorporation of leucine as a major precursor in some trypanosomatids (Ginger et al., 2000). Alternatively, cycloartenol present in *Chilomonas* may have served as an intermediate precursor in the synthesis of ergosterol and brassicasterol in this flagellate (see the discussion above for *Cryptomonas* and *Urotricha*). The presence of cycloartenol in *Urotricha* and *Chilomonas* is an interesting finding and suggests that some protists may be able to synthesize phytosterols. Studies involving a broader number of protists and labeled sterols are still necessary to test this hypothesis.

Cholesterol, the main sterol in most crustaceans (Pakrashi et al., 1989), molluscs (Gordon, 1982), and mammals seems to play a secondary role in the organisms we studied, as suggested by its low relative concentrations in the heterotrophic protists and their diets analyzed here (but see "bacteria + polished rice" and *Cyclidium*, Table 6). The function of cholesterol in regulating membrane fluidity is well established (Bloch, 1992). Nevertheless, it has been generally accepted that phytosterols, detected in large amounts in our protists, also stabilize cell membranes (Moreau et al., 2002). The presence of high phytosterol concentrations in all heterotrophic protist species we analyzed, especially stigmasterol, may suggest analogous regulatory function to cholesterol functions in the cell membranes of heterotrophic protists.

In addition to sterols, the triterpenoids tetrahymanol, diplopterol, and diploptene represented an important fraction of the neutral lipid fraction found in *Cyclidium*. Ciliates as well as bacteria and other microorganisms have been found to be rich in tetrahymanol, a neutral lipid structurally similar to cholesterol, and also in the hopanoids diplopterol and diploptene (see Ourisson et al., 1987 for a review).

These microbial lipids are derived from the tertepene metabolism and are thought to play the same role in prokaryotic membranes that cholesterol does in eukaryotic membranes (Ourisson et al., 1987). Recent studies have demonstrated that some ciliates fed bacteria are able to *de novo* synthesize tetrahymanol (Harvey and McManus, 1991). In the ciliate *Tetrahymena pyriformis*, the presence of cholesterol inhibited tetrahymanol synthesis (Conner et al., 1968), and the authors suggested that tetrahymanol may carry out a function similar to cholesterol in the plasma membrane of *T. pyriformis*. In our study, cholesterol and tetrahymanol were both found in *Cyclidium*. Tetrahymanol synthesis was shown to be hampered by exogenous supply of stigmasterol in the ciliate *Trimyema compressum* (Holler et al., 1993). Stigmasterol was found in low concentrations in *Cyclidium*, but in high concentrations in all other species we investigated (*Balanion*, *Urotricha*, *Chilomonas*), which in turn did not contain tetrahymanol. It could be possible that stigmasterol may hamper tetrahymanol synthesis in *Balanion*, *Urotricha*, and *Chilomonas* but not in *Cyclidium*. Further research is needed in order to determine whether the presence of particular phytosterols interfere with the synthesis of other neutral lipids in protist cells.

Recent studies have revealed the importance of free-living freshwater heterotrophic protists as both consumers of bacteria and algae, and as prey for metazoan grazers (Stoecker et al., 1986, De Biase et al., 1990; Stoecker and Capuzzo, 1990; Wickham et al., 1993; Sanders et al., 1996; Adrian and Schneider-Olt, 1999; Adrian et al., 2001; Burns and Schallenberg, 2001; Mohr and Adrian, 2002a,b). Therefore, heterotrophic protists constitute a trophic link between primary producers and higher trophic level consumers. Depending on their ability to modify the biochemical composition obtained from their diet, heterotrophic protists can affect the transfer of matter and energy to higher trophic levels at an early stage in aquatic food webs. Here we analyzed several different consumer – diet combinations as a first step to understand the biochemical composition of different heterotrophic protists fed a known diet. The next step should include a single protists growing on different food resources to elucidate how the biochemical composition of heterotrophic protists responds to changes in dietary composition. Given the difficulty in cultivating our protist species on different food resources we may probably have to select other protist species, which are easier to culture on different diets.

Overall, our study contributes to the understanding of sterol composition in freshwater heterotrophic protists and suggests that species-specific variation occurs. We have discussed some possible mechanisms which may underlie the mismatch between protist and dietary sterol composition, including direct

assimilation of medium sterols for *Cyclidium* and *Chilomonas*, high ingestion and incorporation efficiencies of dietary sterols as well as sterol synthesis in *Balanion*, *Urotricha*, and in *Chilomonas*. Our results may stimulate further studies involving single labeled sterols in heterotrophic protists and their diet. Such studies are necessary to elucidate the mechanisms of sterol synthesis and metabolism in freshwater heterotrophic protists, thus contributing to our comprehension on the role of heterotrophic protists in the transfer of biochemical matter in aquatic food webs.

4. TROPHIC MODE INFLUENCES FATTY ACID AND STEROL COMPOSITION OF THE CHRYSOPHYTE *OCHROMONAS* SP.

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Keywords: Trophic mode; *Ochromonas* sp.; Fatty Acids; Sterols

ABSTRACT

We investigated the influence of trophic mode – autotrophic, mixotrophic, and heterotrophic – on the fatty acid and sterol composition of the chrysophyte *Ochromonas* sp. Total fatty acid concentrations, especially of polyunsaturated fatty acids, decreased as the trophic mode changed from autotrophic via mixotrophic to heterotrophic. Saturated fatty acids were the most abundant fatty acid class found in heterotrophic *Ochromonas*, whereas polyunsaturated fatty acids were most abundant in autotrophic flagellates. Sterol concentrations were higher in autotrophs and heterotrophs than in mixotrophs, and stigmasterol was the most abundant sterol found in *Ochromonas* of all trophic modes. Discriminant analyses revealed that polyunsaturated fatty acids are the most efficient parameter discriminating among trophic modes. The analyses pointed out single fatty acids, which more efficiently discriminated among trophic modes. Besides disparities in the fatty acid patterns among *Ochromonas* of different trophic modes, differential lipid allocation for survival and cellular division in this protist may occur. Such differential investment in survival or reproduction may dictate the trophic mode, which will dominate within a species, under natural conditions. The dominance of one trophic mode over the other may, in turn, affect the nutritional quality available for predators in nature.

4.1 INTRODUCTION

The fatty acid and sterol composition of non-autotrophic protists have been shown to partially rely on dietary composition (Ederington et al., 1995; chapters 2 and 3). Moreover, species-specific metabolism of fatty acids and sterols may differ among heterotrophic protist species fed a same diet (chapters 2 and 3). However, to which extent the fatty acid and sterol profile of a protist species may be dictated by the trophic mode is still unknown.

Living organisms require energy to drive the chemical reactions necessary for maintenance, growth, and reproduction. This energy is normally obtained by two routes, autotrophy and heterotrophy (Sanders, 1991). Light-energy is absorbed by photoautotrophic organisms, which use this energy to synthesize energy-rich organic molecules during photosynthesis. Heterotrophic organisms, otherwise, relay on the organic composition obtained from ingested particles (phagotrophy) or dissolved matter (osmotrophy). A special strategy of life – mixotrophy – combines both the ability of photosynthesis and the uptake of organic matter (Jones, 1994).

Flagellated protists display all basic trophic strategies known among autotrophy and heterotrophy, and also ensure the largest number of species presenting mixotrophy, especially within *Chrysophyta* (Sanders, 1991). While autotrophs are able to synthesize all biochemical compounds necessary for their metabolic activities, heterotrophs are unable to synthesize most biochemical molecules (Sleigh, 2000). They depend therefore on the uptake of many classes of organic molecules from their environment. Mixotrophs ought to invest in both a photosynthetic apparatus and mechanisms of prey uptake and digestion, which may imply high energetic costs (Tittel et al., 2003), but provide them with an ecological advantage when light or nutrients are limiting (Rothhaupt, 1996a,b). The shift of one growth pathway to another has profound metabolic implications, which drives adaptation (Sleigh, 2000) and may result in altered biochemical composition. Since the nutritional quality of planktonic prey organisms has been associated with the presence of some essential biochemical compounds, such as essential fatty acids and sterols (Brett and Müller-Navarra, 1997; Becker and Boersma, 2003; Von Elert, 2002; Hassett, 2004), the trophic mode may affect the nutritional quality of a prey organism, via changes in the biochemical composition.

The chrysophyte *Ochromonas* sp. is a common flagellate in many aquatic systems with a broad range of habitats. It is also able to cope with extreme environments such as acidic water bodies, e.g. mining lakes (Wollmann et al., 2000). *Ochromonas* species have been commonly identified as mixotrophs in both marine and freshwater plankton (Sanders et al., 1990). Besides that, *Ochromonas* has been successfully cultured as autotrophs and heterotrophs (Tittel et al., 2003; this study).

In the present study we investigate whether the fatty acid and sterol profile of *Ochromonas* sp. depends on their trophic mode. We discuss the differences in fatty acid and sterol composition among *Ochromonas* sp. cultured autotrophically, mixotrophically, and heterotrophically in light of metabolic features of the flagellates and consider the role of such differences in an ecological perspective.

4.2 METHODOLOGY

4.2.1 Cultures

Ochromonas was isolated from a mining lake (L 111) in Lausitz, Germany. L 111 is characterized by a low pH of ca. 2.7 and high concentrations of iron and sulphate (Wollmann et al., 2000). *Ochromonas* sp. is the dominant phytoflagellate in the lake and has its population maximum in summer in the epilimnion (Tittel et al., 2003; Kamjunke et al., in press). The prevailing trophic mode in the field is heterotrophy, potentially subsidised by autotrophy (Tittel et al., 2003). *Ochromonas* was cultured in a medium reflecting the ion composition of lake L 111 (Bissinger et al., 2000) at a temperature of 20 °C. Three different kinds of cultures were set up in duplicate. For autotrophic growth, the cultures were kept in the light at a light : dark regime of 16:8 h. Although the cultures were not axenic, bacteria growth and abundance were low as the medium was free of organic carbon. For mixotrophic growth, the cultures were under the same light conditions as for autotrophic growth and fed bacteria isolated from L 111. For heterotrophic growth the cultures were treated in the same way but kept in the dark. Bacteria cultures were cultured in the same medium as for *Ochromonas* sp., except for the addition of glucose to promote bacterial growth. Throughout the text mixotrophy is used for describing the strategy of combining phototrophy and heterotrophy.

4.2.2 Sample Preparation

Flagellate and bacteria cultures were filtered in replicate on pre-combusted glass fibre filters. GF/C filters (Whatman) were used for collecting *Ochromonas* samples and GF/F filters (Whatman) were used for collecting bacterial samples. The density of flagellates and bacteria was determined in both the cultures and the filtrates, and the difference was calculated for determining the biomass captured on the filter. Samples were fixed with Lugol´s Iodine and flagellates were counted with an inverted microscope. Bacteria were stained with acridine orange and counted using a fluorescent microscope (Zeiss, Axioscope 2). The carbon content of the bacteria was calculated from size measurements according to Simon and Azam (1989). *Ochromonas* was measured with a computer-aided image analysis (TSO Thalheim). The volume of the cells was calculated assuming a rotational ellipsoid, and converted into carbon units assuming 0.23 pgC per µm³ cell volume (Kamjunke et al., in press).

4.2.3 Fatty Acid and Sterol Analysis

Filters containing autotrophic, mixotrophic, and heterotrophic *Ochromonas* as well as the bacterial diet were extracted in chloroform-methanol 2:1, v/v solution (Folch et al., 1957) and homogenized by sonication for 5 min at 5000 cycles per min (Ultrashall-Desintegrator USD 20, VDE Wiss. Gerätebau, Berlin). After sonication, an internal standard was added to the samples (tricosanoic acid, 0.2 mg mL^{-1} for fatty acids, and 5α-cholestane, 0.2 mg mL^{-1} for sterols). The samples were then allowed to extract for 3 h at 20°C. After extraction, samples were dried under nitrogen flux and promptly stored at –20°C until analysis.

For fatty acid analysis, fatty acid methyl-esters (FAME) were formed by addition of 5 mL sulphuric acid (5% v/v) and heating the samples for 4 h at 80°C (Weiler, 2001). An aliquot of 0.2 µL of the samples was finally injected into a Varian Star 3600 CX series gas chromatograph, equipped with a fused silica capillary column (Omegawax 320, SUPELCO, 30 m x 0.32 mm). The following heating program was applied: initial temperature of 180°C (2 min), subsequent heating at 2°C min-1 to 200°C, which was held isothermally for 33 min. Injector and FID detector temperatures were 250°C and 260°C, respectively. Helium was used as a carrier gas. FAMEs identification were made by comparing the retention times with retention times of a calibration standard solution (Supelco FAME Mix 47885–4, PUFA Nr. 3–47085–4 and PUFA Nr. 1–47033) and quantified by comparing the peak areas with the peak area of the internal standard. For presentation, we selected some fatty acids from the total measured pool. It should be kept in mind

that the SAFA, MUFA, and PUFA sums refer to the whole pool of measured fatty acids, i.e. including those not presented.

For sterol analysis extracts were subjected to alkaline hydrolysis (saponification) by the addition of 5 mL 1 N potassium hydroxide (KOH) solution in 80% methanol (MeOH), followed by heating at 80°C for 30 min (Gordon and Collins, 1982). Free neutral lipids were separated and subsequently silylated by adding 25 μL Bis (trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane (BSTFA), 75 μL pyridine, and heating the sample at 60°C for 30 min (Breteler et al., 1999). Sterol silylether derivatives were analyzed using a gas chromatograph Agilent 6890 equipped with a mass selective detector Agilent 5973-N and a fused silica capillary column HP-5MS (60 m x 0.32 mm x 0.25 μm). The carrier gas (helium) was held constant at 1.3 mL min^{-1}. The temperature of the PTV (programmed temperature vaporization) inlet – operating in splitless mode – was 300°C (initial temperature 100°C, 720°C min^{-1}). The temperature of the detector interface was 280°C. The following temperature program was employed: 150°C initial temperature for 3 min, than heating at 4°C min^{-1} up to 300°C, and maintained for 40 min. Sterol silylether derivatives were identified by their retention times and their mass spectra in full scan mode (SCAN) previously calibrated with individual sterol standards (Sigma-Aldrich). The generated mass spectra were compared with mass spectra of a self-generated spectra library (Agilent Chemstation). Sterol silylether derivatives were quantified by selective ion monitoring (SIM) at the two most intensive ions at the molecular ion cluster. Calibration curves ranged between 0.04 and 0.4 μg sterol per mL injected sample. Sterols are provided as percentages of the total sterol pool (relative amounts) and as absolute concentration per carbon biomass, whenever a commercial standard was available. We refer to the sterols by their trivial names along the text and at the tables, in order to facilitate reading and comparisons. However, we indicated the classic nomenclature when first referencing to the sterol common name (there are currently two main nomenclatures following the IUPAC-IUB recommendations; see Moreau et al. [2002] for a list of synonyms currently used).

4.2.4 Statistical Analyses

Differences in the fatty acid and sterol composition among *Ochromonas* samples of different trophic modes were tested with one-way ANOVA followed by a pairwise post-hoc Tukey – *HSD* test (Statistica for Windows, version 5.01, Stat Soft). Percentage data were arcsin root square transformed prior to ANOVA. To single out the fatty acids and sterols that mostly differed among *Ochromonas* of different trophic modes we performed discriminant analyses considering the

absolute concentrations of the biochemical compounds. Discriminant analysis is an ordination method used to determine which single variables within a pool of variables better discriminate between two or more defined groups. In our case, the analyses were performed to determine which fatty acids and sterols (variables) better separated *Ochromonas* of different trophic modes (groups). In the case of a multiple group stepwise discriminant analysis (3 different groups – autotrophs, mixotrophs, and heterotrophs), the ultimate calculation correspond to a canonical correlation analysis, which provides the successive functions and canonical roots containing the variables mostly responsible for separating the groups. For the fatty acids we performed the discriminant analyses separately for saturated, monounsaturated, and polyunsaturated fatty acids. Although we do not present the entire pool of fatty acids measured, all fatty acids were considered for the statistical analyses. All statistical procedures were run in Statistica for Windows (version 5.01, Stat Soft).

4.3 RESULTS

4.3.1 Fatty Acids

Fatty acid relative composition varied among *Ochromonas* of different trophic modes (Fig. 1). Autotrophic *Ochromonas* exhibited higher percentages of polyunsaturated fatty acids (44%) than mixotrophs (15%) and heterotrophs (16%) (one-way ANOVA $F=38.77$, $P<0.01$). Percentages of monounsaturated acids were similar in autotrophic (21%), mixotrophic (28%), and heterotrophic flagellates (31%) (one-way ANOVA $F=6.52$, $P=0.13$). Lower percentages of saturated fatty acids were found in autotrophic (28%) when compared to mixotrophic (64%) and heterotrophic (53%) flagellates (one-way ANOVA $F=29.79$, $P<0.01$).

Mixotrophic and heterotrophic *Ochromonas* showed both higher percentages of saturated and lower percentages of monounsaturated fatty acids than those observed in the bacterial diet (one-way ANOVA $F_{SAFA}=13.07$, $P<0.01$; $F_{MUFA}=27.38$, $P<0.01$). The total percentage of polyunsaturated fatty acids in the flagellates reflected the total percentage found in the bacterial diet (one-way ANOVA $F=0.17$, $P=0.84$; Fig.1).

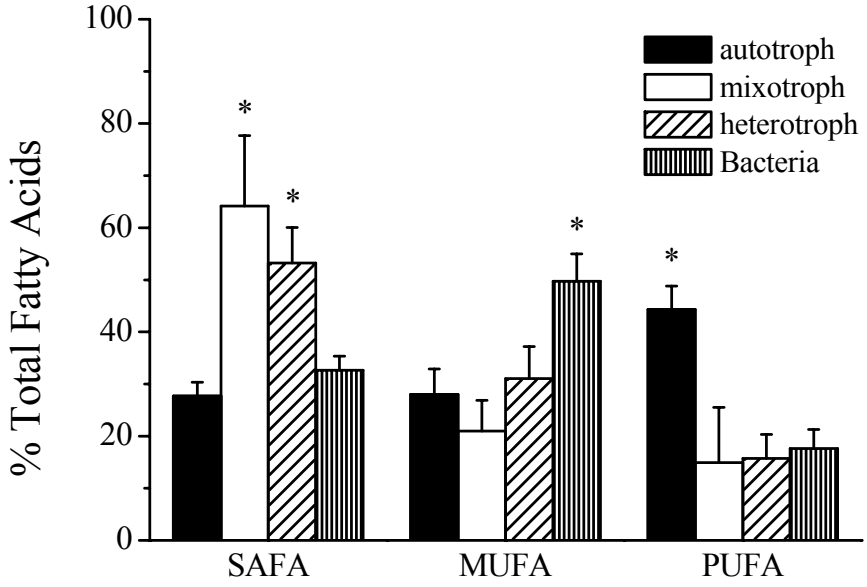

Fig. 1 – Percentages of saturated (SAFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids in the total fatty acid content of autotrophic, mixotrophic, and heterotrophic *Ochromonas* sp. as well as in the bacterial diet of heterotrophic and mixotrophic *Ochromonas* sp. Significant differences at 95% confidence are represented by an astherisk.

Differences in the absolute concentration of individual fatty acids also reflected the trophic mode (Table 7). Autotrophic and mixotrophic flagellates had much higher concentrations of the saturated acids 16:0, 17:0, and 18:0 than heterotrophic flagellates (Table 7). The acid 20:0 was not found in heterotrophic flagellates, which in turn had minor amounts of 19:0, not detected in their bacterial diet. The concentrations of individual monounsaturated fatty acids such as 15:1, 16:1ω5, and 17:1 were higher in both autotrophic and mixotrophic flagellates than in heterotrophic ones (Table 7). Heterotrophic flagellates were rich in the monounsaturated fatty acid 18:1ω9 also found in high concentrations in the bacterial diet. The acid 22:1ω9 was only found in autotrophic and mixotrophic flagellates. The highest discrepancies were observed for individual polyunsaturated fatty acids. Heterotrophic flagellates had no polyunsaturated acid with a carbon-chain longer than 18 carbons, and the absolute concentrations of all polyunsaturated acids present reflected the concentrations found in the bacterial diet (Table 7). Autotrophic and mixotrophic flagellates in turn contained high concentrations of long-chain PUFA (more than 20 carbon-atoms chain length), especially of 20:3ω6, 21:5ω3 (DPA), and 20:5ω3 (EPA) in case of mixotrophs,

and 22:6ω3 (DHA) in the case of autotrophs (Table 7). Total polyunsaturated fatty acid concentrations in autotrophic and in mixotrophic flagellates were around 18 and 7 times higher than those found in heterotrophic flagellates. Ratios of ω6 to ω3 fatty acids were similar among mixotrophic and heterotrophic flagellates, but lower in autotrophic flagellates (one-way ANOVA F=7.51, $P<0.01$; Table 7).

The discriminant analyses run separately for saturated, monounsaturated, and polyunsaturated fatty acids provided the fatty acid combinations which discriminated most efficiently among *Ochromonas*' trophic mode (Fig. 2).

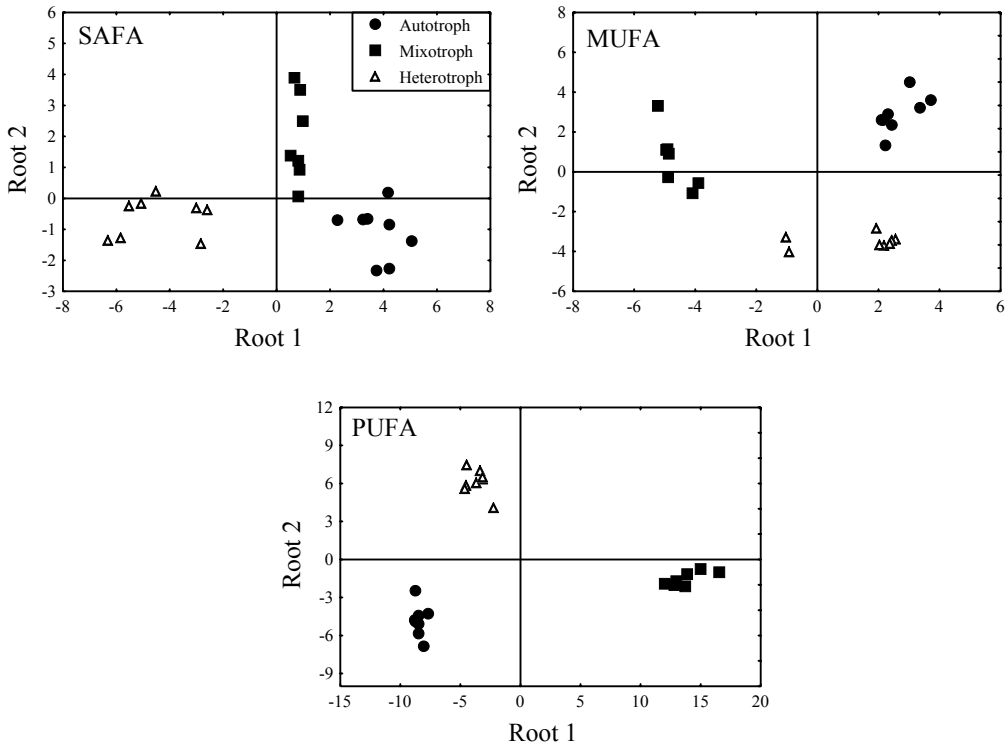

Fig. 2 – Canonical roots provided by discriminant analyses run for saturated (SAFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) of autotrophic (●), mixotrophic (▲), and heterotrophic (□) *Ochromonas* sp. See text and Table 8 for the fatty acids discriminating within each root.

For saturated fatty acids only the first root was significant (Wilk's Lambda = 0.023; F = 6.16, $P<0.001$). The three trophic modes were separated at this root, but higher differences were observed between autotrophs and heterotrophs, whereas mixotrophs occupied an intermediate position (Fig. 2). The separation was mostly due to differences in 16:0, 18:0, 20:0, 21:0, and 24:0 (Table 8). The discriminant analysis on monounsaturated fatty acids provided two significant roots (Wilk's Lambda=0.008; F=6.01, $P<0.001$). The first root primarily discriminated mixotrophs from the other groups, due to differences in the fatty

acids 15:1 and 20:1ω9 (Table 8). The second root better separated autotrophs from heterotrophs (Fig. 2), due to differences in the monounsaturated 17:1, 20:1ω11, and 22:1ω9 (Table 8). Polyunsaturated fatty acid were the most efficient variables in separating the three trophic modes of *Ochromonas*, as revealed by the two significant roots extracted by the discriminant analysis (Wilk's Lambda=0.0004; F=31.09, $P<0.001$). The first root discriminated mixotrophs from the other trophic groups (Fig. 2). Differences in the polyunsaturated 20:3ω3, 16:3ω4, 18:3ω3, 18:3ω4, and EPA were responsible for separating mixotrophs at this level (Table 8). The second root better separated heterotrophs from autotrophs due to differences in the polyunsaturated acids 16:2ω4, DHA, 18:3ω6, 18:4ω3, 20:3ω6, 22:5ω3, DPA, and 20:4ω3 (Table 8).

4.3.2 Sterols

Total sterol concentration did not differ significantly among *Ochromonas* of different trophic mode, although significant differences were found for single sterols (Table 9). Overall, mixotrophic *Ochromonas* had the lowest absolute sterol concentrations among the flagellates (ANOVA, all $P<0.05$; Table 9). The only exception was stigmasterol, which was similar in mixotrophic and autotrophic flagellates (ANOVA F=4.84, $P<0.05$). Squalene, the sterol precursor in all sterol biosynthetic pathways, was found in all *Ochromonas* in significant different concentrations (ANOVA F=48.97, $P<0.001$). Interestingly, although absolute concentrations in mixotrophic *Ochromonas* were generally lower than in autotrophs and heterotrophs, the relative amounts did not differ with trophic mode (Fig. 3).

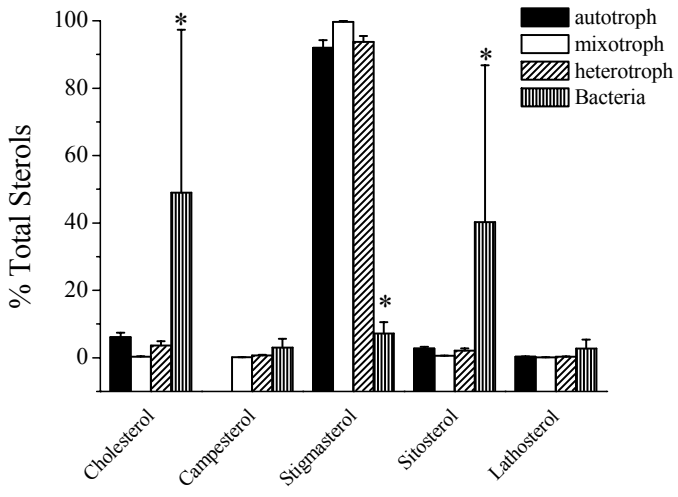

Fig. 3 – Sterol percentages in autotrophic, mixotrophic, and heterotrophic *Ochromonas* sp. as well as in the bacterial diet of heterotrophic and mixotrophic *Ochromonas* sp. Significant differences at 95% confidence are represented by an astherisk.

Hence, stigmasterol was the predominant sterol in all *Ochromonas*, covering up to 98% of the total sterol pool. In contrast to the relative sterol composition of heterotrophic and mixotrophic flagellates, the bacterial diet presented sitosterol and cholesterol as predominant sterols (Fig. 3). Non-sterol tertepenoids were not observed in *Ochromonas* or in the bacterial diet.

Two significant roots were extracted from the discriminant analysis run for the sterol absolute concentrations in *Ochromonas* of different trophic modes (Wilk's Lambda=0.151; F=4.73, $P<0.001$) (Fig. 4). The first root separated autotrophs from the other trophic modes (Fig. 4), due to differences in cholesterol and sitosterol concentrations (Table 10). The second root discriminated between heterotrophs and mixotrophs (Fig. 4), mainly due to differences in campesterol and lathosterol (Table 10).

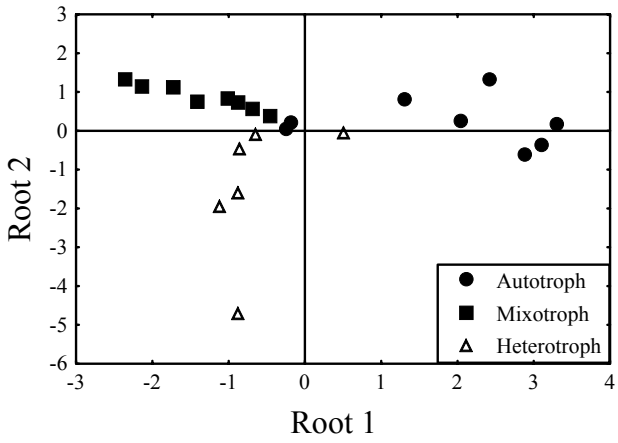

Fig. 4 – Canonical roots provided by discriminant analyses run for the sterol composition of autotrophic (●), mixotrophic (▲), and heterotrophic (□) *Ochromonas* sp. See text and Table 10 for the sterols discriminating within each root.

4.4 DISCUSSION

The fatty acid profiles in *Ochromonas* sp. reflected the trophic mode of the flagellates, with concentrations of polyunsaturated fatty acids decreasing when the growth pathway changed from autotrophic via mixotrophic to heterotrophic. A similar pattern was found for several other protists (Vazhappilly and Chen, 1998; Heifetz et al., 2000; Poerschmann et al., 2004), which probably reflect metabolic adaptations imposed to organisms growing on fluctuating environmental conditions of light and nutrients (Sanders et al., 2001; Hochachka and Somero,

2002). In a study carried out with the chlorophyceae *Chlamydomonas* sp., also isolated from the mining lake L111, a principal component analysis (PCA) proved to be a good tool for separating groups of fatty acids distinguishing among autotrophic, mixotrophic, and heterotrophic modes (Poerschmann et al., 2004). By using the discriminant analysis we did not only show that polyunsaturated fatty acids are a better indicator of the trophic mode than saturated and monounsaturated fatty acids, as suggested by the PCA in the aforementioned study, but we were also able to identify single polyunsaturated fatty acid accounting for the best separation of *Ochromonas* grown under different trophic conditions. For instance, the first canonical root of the DA for polyunsaturated fatty acids showed that fatty acids of 18 and 20 carbon-atoms belonging to the ω3 family (e.g. 18:3ω3, 20:3ω3) were the most appropriate to separate mixotrophs from autotrophs. On the other hand, long-chain acids with 20 or more carbon-atoms mainly separated heterotrophs at the second root of the discriminant analysis. Heterotrophic flagellates, in contrast to autotrophs or mixotrophs, are possibly unable to elongate the hydrocarbon chain, given the absence of polyunsaturated fatty acids with more than 18 carbons in heterotrophic *Ochromonas*.

According to the discriminant analysis, long-chain polyunsaturated fatty acids provided the best discrimination between autotrophs and mixotrophs, suggesting that polyunsaturated fatty acids may be a better indicator of the trophic mode, than saturated or monounsaturated acids. This is not surprising, since energetic costs to build polyunsaturated fatty acids are much higher than those for monounsaturated or saturated ones. Also the presence of a broader number of accessory enzymatic systems is necessary to insert double bounds into the hydrocarbon chain (Ratledge and Wilkinson, 1988; Stryer, 1995). It is reasonable to think that mixotrophs will preferentially use carbon compounds such as glucose obtained from the medium or through bacterial consumption to build structural biomolecules, such as carbohydrates and phospholipids, and to generate energy, instead of synthesizing complex long-chain polyunsaturated fatty acids. On the other hand, the ability to do photosynthesis enables mixotrophs to synthesize some compounds, which can be stored as energy reserve molecules to be used at times of light limitation (e.g. EPA, DHA, and DPA in this study). Some authors suggest that the prime purpose of mixotrophy is to obtain carbon for growth (Bird and Kalff, 1986; Caron et al., 1990; Jones et al., 1993). If so, carbon obtained by phagotrophy may be primarily used for building structural biomolecules such as carbohydrates and phospholipids, whereas photosynthesis may be responsible for generating

substrates for the synthesis of more complex energetic biomolecules, such as triglycerydes.

The higher percentages of saturated fatty acid in mixotrophs and heterotrophs than in autotrophs (Fig. 1) probably reflected the phagotrophic mode of nutrition, with bacterial fatty acids, mostly saturated ones, covering a large fraction of the fatty acid pool of mixotrophs and heterotrophs. A number of factors are expected to affect the degree of phagotrophy including light, dissolved organic carbon concentration, and food concentration (Sanders et al., 1990; Jones et al., 1993). On the other hand, loss of chlorophyll occurs with increased dependency on phagotrophy (Caron et al., 1990; Sanders et al., 1990). The overall lower content of fatty acids found in heterotrophic species compared to the corresponding mixotrophs and autotrophs may indicate changes in extra-chloroplastic membranes as well as loss of chloroplast lipids (Lösel, 1988).

There is a debate as to why flagellates graze bacteria rather than just carring out photosynthesis. Some workers argue that it is a mean of acquiring nutrients for photosynthesis during periods of limitation (Nygaard and Tobiesen, 1993). However, under optimal culture conditions, nutrients are provided in sufficient quantities to support cellular growth and reproduction. Nevertheless, mixotrophic flagellates in our cultures adopted both strategies, as reflected by their fatty acid composition. Mixotrophs contained high concentrations of saturated fatty acids, a typical pattern for organisms feeding on bacteria, but they also yield polyunsaturated fatty acids, which were not found in heterotrophs. Hence, light should be the most important factor driving metabolic patterns of fatty acid synthesis and allocation in autotrophs and mixotrophs. The enzyme acetyl CoA carboxylase catalyses the first step of fatty acid synthesis and its activity depends on both light and the rate of fatty acid synthesis in the chloroplast of higher plants (Post-Beitenmiller et al., 1992). *De novo* synthesis of lipids in plants also depends on NADPH generated in the light reactions of photosynthesis. Hence it is expected that lipid synthesis rate and the relative allocation of photosynthates into lipids both tend to increase with incubation irradiance (Wainman and Lean, 1992). Our findings on fatty acid composition support this transition from heterotrophy to autotrophy. It is important to note that fatty acid composition is strongly influenced by external temperatures, due to the function of these lipids in regulating membrane fluidity as an adaptation mechanism to temperature shifts (Davidson, 1991). At low temperatures, the fatty acid pattern may be primarily dictated via membrane fluidity regulation rather than by the trophic mode (Poerschmann et al., 2004).

The sterol pattern identified for *Ochromonas* sp., with stigmasterol as the predominant sterol, was already described for *Ochromonas danica* (Halevy et al., 1966), *Ochromonas malhamensis* (Avivi et al., 1967), and *Ochromonas sociabilis* (Goodwin, 1974). Gershengorn et al. (1968) found porifesterol (the 24β-epimer of stigmasterol) to be the predominant sterol in *O. malhamensis* (98% of the total sterol pool). The presence of sitosterol (or its 24β-epimer – clionasterol), cholesterol, brassicasterol, and ergosterol was also shown in previous studies (Gershengorn et al., 1968; Tsai et al., 1975). Interestingly, except for stigmasterol, sterol composition in mixotrophic flagellates did resemble that of bacteria, whereas autotrophs and heterotrophs showed rather similar composition. Possibly, mixotrophy leads to a shortage in sterol synthesis and storage. The high efforts of a mixotroph to run both an autotrophic and a phagotrophic metabolism may cause higher basic metabolic costs and limit reproductive rates as compared to more specialized organisms, such as heterotrophs and autotrophs (Rothhaupt, 1996a). One of those metabolic costs may be decreased synthesis rates of some sterols, which may have analogous function to other molecules, such as fatty acids. In mixotrophic *Ochromonas*, sterol synthesis may have been mostly directed to stigmasterol synthesis, as stigmasterol concentrations in mixotrophs were similar to those found in autotrophs and heterotrophs. Stigmasterol has been found to be a major sterol in phagotrophic and phototrophic protists (chapter 3, Tables 5 and 6), suggesting its importance for protists. Apart from stigmasterol synthesis, synthesis of other sterols may have been reduced to the minimum necessary to guarantee cellular functioning in mixotrophs.

Differences in cholesterol concentrations separated autotrophs from heterotrophs, whereas the phytosterol sitosterol separated autotrophs from mixotrophs. Cholesterol synthesis requires molecular oxygen and NADPH (Stryer, 1995) and is thus supposed to be strongly regulated by photosynthesis and respiration rates (Stryer, 1995). On the other hand, synthesis of phytosterols is regulated by the presence of some precursors, which differ from those needed for cholesterol synthesis (Moreau et al., 2002). The presence and abundance of such phytosterol precursors may have accounted for the observed differences in sitosterol concentrations in autotrophs versus mixotrophs. Discriminant analysis on sterols was by far less efficient to differenciate trophic mode of *Ochromonas* under laboratorial conditions when compared to the analysis on polyunsaturated fatty acids. This means that sterol concentration may be an inadequate parameter to distinguish trophic mode at non-limiting nutrient conditions. This hypothesis is supported by the finding that, despite differences in sterol absolute concentrations, relative amounts remained the same, independently of the trophic mode (Fig. 3). It

remains the question, how efficient are these lipids in differentiating trophic mode under conditions of nutrient limitation.

Interestingly, discriminant analyses on monounsaturated and polyunsaturated fatty acids separated mixotrophic flagellates from the other trophic modes, while sterols separated autotrophs from heterotrophs and mixotrophs at the first root. This may be an effect of differentiated patterns of lipid synthesis and allocation in *Ochromonas* growing under different trophic modes. Given the differences in function and biosynthetic pathways between fatty acids and sterols, these lipid classes may be expected to respond differently to environmental factors such as light and nutrients (Parish and Wangersky, 1987; Smith and D'Souza, 1993).

Lipid allocation may reflect differential ecological strategies in protists. Higher percentages of the polyunsaturated fatty acids 20:4ω6 and 18:3ω3 have been found to be allocated into triglycerides instead of phospholipids in autotrophic protists of the genus *Cryptomonas* (Boëchat, unpublished data). In heterotrophic protists (e.g. *Cyclidium* sp) higher percentages of these PUFA were found in the phospholipid fraction (Boëchat, unpublished data). Triglycerides are very important energy storage molecules, whereas phospholipids are essential components of membranes. A differential allocation of PUFA into triglycerides or phospholipids may suggest a differential investment into reproduction or growth in protists. Under light limitation lipid allocation in membranes may be stimulated to enable higher division rates. In fact, heterotrophic growth of *Ochromonas* did not deviate from mixotrophic growth (0.32 ± 0.06 day^{-1} and 0.27 ± 0.02 day^{-1}, respectively; mean ± SE) whereas autototrophic growth was close to zero (0.07 ± 0.07 day^{-1}) under sufficient nutrient conditions (Tittel et al., 2003). Autotrophy would than imply lower division rates and high energy allocation if nutrients are not limiting. The ecological theory predicts that specialization should be the most successful strategy for survival under non-limiting conditions (Rothhaupt, 1996a). The question remains to be answered, whether survival may represent a more important investment than high division rates for autotrophic protists under non-limiting conditions in nature.

The fact that differences in the fatty acid and sterol composition found for *Ochromonas* sp. were probably caused by the trophic mode may strongly affect the nutritional quality of this protist as prey. The fact that one trophic mode may predominate over the others in extreme ecosytems, such as mixotrophy in antartic polar lakes (Bell and Laybourn-Parry, 2003), may have profound nutritional consequences for predators living in such ecosystems.

5. Evidence For Biochemical Limitation On Reproduction Patterns Of The Rotifer *Keratella quadrata* Fed Freshwater Heterotrophic Protists

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Abstract

Recent studies have demonstrated that the trophic mode shapes the nutritional quality of heterotrophic protists for rotifers. However, the biochemical factors that determine protist nutritional quality for rotifers remain unclear. We evaluated population growth rates and egg numbers of the rotifer *Keratella quadrata* fed either algivorous or bacterivorous protist species. The cryptomonad *Cryptomonas phaseolus*, considered a good quality prey, was used as control. Population growth rates and egg numbers of *K. quadrata* were correlated with single biochemical compounds (fatty acids, amino acids, sterols) of the heterotrophic protists. Feeding on algivores and the alga *Cryptomonas phaseolus* resulted in positive population growth rates and high egg numbers of *K. quadrata*, whereas feeding on bacterivores resulted in moderate egg production but no population growth. *K. quadrata* egg numbers were significantly correlated with protist biochemical composition, including several polyunsaturated fatty acids, sterols, and the amino acid leucine. No significant relationship was observed between growth rates of the rotifers and protist biochemistry, suggesting that rotifer growth and reproduction probably have different nutritional requirements. Factors apart from the biochemical components analysed, like nutrient ratios, vitamins, trace-elements, as well as synergetic and antagonistic interactions between chemical substances may have additionally influenced protist nutritional quality for *K. quadrata*.

5.1 INTRODUCTION

Rotifers represent an important fraction of the zooplankton biomass in lakes (Arndt, 1993), and their prey size spectrum (< 1 – 200 µm, Pourriot, 1977; Bogdan and Gilbert, 1987) covers a wide range of microbes, including bacteria and heterotrophic protists. In lakes during spring, the abundance of rotifers peaks shortly after the abundance peak of heterotrophic protists (Sommer et al., 1986); it is well known that predation by rotifers may potentially regulate protist population *in situ* (Bogdan et al., 1980; Carrick et al., 1991; Gilbert and Jack, 1993; Mohr and Adrian, 2002a). However, despite the ecological significance of heterotrophic protists – rotifer interaction, it is still not fully understood how heterotrophic protists contribute to growth and reproduction of rotifer populations.

The influence of protist-prey on the life history of rotifers has been recently reported (Gilbert and Jack, 1993; Mohr and Adrian, 2001; Weisse and Frahm, 2001; Mohr and Adrian, 2002b). Enhanced reproduction and growth rates of *Brachionus calicyflorus* Pallas 1766 were observed when offering the rotifers a mixture of algae and algivorous ciliates. However, no positive effect was reported when the rotifers were offered a mixture of algae and bacterivorous ciliates (Mohr and Adrian, 2002b). Moreover, enhanced growth of rotifer populations of the genus *Keratella* was observed when the rotifers were fed a mixture of autotrophic cryptophytes and algivorous ciliates (Weisse and Frahm, 2001). Taken together, these results suggest that the diet source of heterotrophic protists – algae or bacteria – shapes their nutritional quality for rotifers. Indeed, previous studies have shown that dietary biochemical composition influences protist fatty acid and amino acid (chapter 2) as well as the sterol composition (chapter 3). Nevertheless, even when fed the same prey the biochemical composition of predators can differ species-specifically. Such disparities in the biochemical composition between heterotrophic protists and their diet suggest species-specific differences in protist metabolism, which could additionally affect nutritional quality of heterotrophic protists for mesozooplankton predators.

Studies on the nutritional quality of planktonic prey organisms have emphasized the importance of essential biochemical compounds in promoting growth and reproduction of zooplankton (e.g. Ahlgren et al., 1990; Brett and Müller-Navarra, 1997; Weers and Gulati, 1997; Becker and Boersma, 2003). Among the substances that have received considerable attention are highly unsaturated fatty acids (HUFA) of the ω3 and ω6 families, like eicosapentaenoic acid (20:5ω3, EPA) and docosahexaenoic acid (22:6ω3, DHA) as well as some polyunsaturated

fatty acids (PUFA), like arachidonic acid (20:4ω6) and linoleic acid (18:2ω6). Enhanced growth and reproduction of cladocerans, especially *Daphnia*, have been associated with higher contents of HUFA and PUFA in cultured prey organisms (e.g. DeMott and Müller-Navarra, 1997; Park et al., 2002; Becker and Boersma, 2003) and in lake seston (e.g. Müller-Navarra et al., 2000; Park et al., 2003; Müller-Navarra et al., 2004).

Along with essential ω3 and ω6 fatty acids, amino acids (Kleppel et al., 1998; Guisande et al., 2000), and more recently the sterol composition of prey organisms (Von Elert et al., 2003; Hasset, 2004), have been reported to be nutritionally important for zooplankton predators. Other studies have suggested that prey mineral composition and stoichiometry, especially the P:C and N:C ratios, may play a more important role in zooplankton nutrition (e.g. Sterner et al., 1992; DeMott et al., 1998; Plath and Boersma, 2001).

Here, we investigated how algivorous and bacterivorous protists contribute to the nutrition of rotifers, and specifically, which biochemical components of the protists may be responsible for their quality as prey for rotifers. For these purposes, we performed population growth and reproduction experiments using the common species *Keratella quadrata* as predator and two algivores and two bacterives as prey. *Cryptomonas phaseolus*, considered a good quality alga, as well as treatments without prey served as experimental controls. The biochemical composition of the heterotrophic protists (analysed in the chapters 2 and 3) was correlated with the results on population growth rates and egg numbers of *K. quadrata*. To our knowledge, this study is the first to consider a wide range of biochemical parameters and to explore nutritional quality of heterotrophic protists for rotifers. Our study highlights the importance of heterotrophic protists as prey for rotifers and suggests some biochemical factors influencing their nutritional quality for *K. quadrata*.

5.2 METHODOLOGY

5.2.1 Cultures

The algivorous ciliates *Balanion planctonicum* (3256 ± 1331 μm^3, average biovolume ± SD) and *Urotricha farcta* (2778 ± 1707 μm^3) were cultured in WC medium (Guillard and Lorenzen, 1972) in frequently diluted batch cultures

incubated at 17 ± 1°C under a 12:12 h light : dark regime. The ciliates were fed the cryptomonad *Cryptomonas phaseolus* (392 ± 125 μm^3), obtained from the Algal Collection of the University of Göttingen, Germany, and cultured in WC medium at 17 ± 1°C under a 16:8 h light : dark regime.

The bacterivorous ciliate *Cyclidium* sp. (1315 ± 617 μm^3) and the flagellate *Chilomonas paramecium* (403 ± 288 μm3) were cultivated in Volvic mineral water (a spring water, poor in minerals, sold worldwide by Société des Eaux de Volvic, Puy-de-Dôme, France) and were fed a bacterial assemblage grown on previously autoclaved rice corns. Bacteria generally comprised around 20 – 40 % of the total carbon content in the cultures of bacterivores. The cultures were kept at 18 ± 1°C under a 16:8 h light : dark regime.

The rotifer *K. quadrata* was originally isolated from the lake Müggelsee in Berlin, Germany, and cultivated in WC medium in frequently diluted batch cultures incubated at 18 ± 1°C under a 16:8 h light : dark regime. The rotifers were routinely grown on the cryptomonad *C. phaseolus*. Henceforth, prey and predator species are referred to by their genus names only.

5.2.2 Reproduction and Population Growth Experiments

We conducted a series of feeding experiments in which reproduction patterns and population growth of *Keratella* fed algivorous and bacterivorous protists were investigated. By choosing protist species of similar size, shape and mobility, we have minimized the influence of morphological and behavioural features of the prey on population growth and egg numbers produced by *Keratella*. Functional response experiments for *Keratella* fed each protist were conducted previously to the feeding experiments (data not shown) in order to determine the ILL (incipient limiting level) for *Keratella* fed each protist, which was the basis for determining prey concentrations used during the feeding experiments. Through the functional experiments we assured that the rotifers indeed ingest the heterotrophic protists. Four prey treatments were conducted, each one with five replicates: Treatment 1: *Balanion* + *Cryptomonas* (2 x 10^3 *Balanion* cells mL^{-1} + 5 x 10^3 *Cryptomonas* cells mL^{-1}); Treatment 2: *Urotricha* + *Cryptomonas* (2 x 10^3 *Urotricha* cells mL^{-1} + 5 x 10^3 *Cryptomonas* cells mL^{-1}); Treatment 3: *Cyclidium* + bacterial assemblage (3.5 x 10^3 *Cyclidium* cells mL^{-1}); and Treatment 4: *Chilomonas* + bacterial assemblage (5 x 10^3 *Chilomonas* cells mL^{-1}). Control treatments with *Cryptomonas* as sole prey (5 x 10^3 cells mL^{-1}) as well as controls without prey were run in parallel.

Bacterivorous protists were separated from their bacterial prey by filtering the cultures through a 10 μm net. Due to their similar size dimensions, we could not separate algivorous ciliates from their algal food, which explains why treatments 1 and 2 contained mixed diets of ciliates and *Cryptomonas*. To test whether differences in rotifer growth rates and egg production in these treatments were due to the additional presence of the algivorous ciliates, algal density in the treatments was kept as in the control treatment with *Cryptomonas* as sole prey in densities above the ILL (5 x 10^3 cells mL^{-1}). As both the ciliates and the algae were offered in concentrations above the incipient limiting level (ILL, 0.8 – 1.5 mgC L^{-1}) this excluded a major limitation by food quantity instead of food quality. It is important to note, that the ILL for *Keratella* fed algivorous ciliates was adequately estimated using cilate food suspensions containing the same algal densities as used for the treatments with *Cryptomonas* as sole prey. Under such conditions, *Keratella* efficiently ingested the algivorous ciliates (in a rate of 8 ciliates $rotifer^{-1}$ h^{-1}), even though the algal density present was sufficient to sustain rotifer's populations.

Feeding experiments were run in macrotiter plates (final volume per chamber 12 mL), incubated at 18 ± 1°C under a 16:8 h light : dark regime. Twenty well-fed *K. quadrata* individuals without eggs were initially transferred into each chamber. The rotifers were separated from their algal diet by filtration through a 70 μm mesh and resuspended in WC medium 12 h prior to the experiments. The addition of cetyl-alcohol pellets reduced the superficial tension in the experimental chambers, thus preventing rotifer mortality through adherence to the surface film. The rotifers received fresh prey suspensions daily over a 5 d experimental period. As a common practice in such feeding experiments, the predators are daily transferred into a new chamber with fresh prey suspension. However, due to the high fragility of *Keratella* to manual handling, we decided not to remove them from the experimental chambers. Instead, we added fresh prey suspensions to the chambers, starting the experiments with an initial volume of 4 mL and adjusting the prey densities daily to a final volume of 12 mL at day 5. Rotifers and eggs were enumerated at x20 magnification (stereoscope). Population growth rates (r) of the rotifers were calculated assuming exponential growth according to:

$$r = \frac{\ln(N_t) - \ln(N_{t-1})}{\Delta t}$$

Where N_t is the number of rotifers at the end of the experimental time interval (Δt) and N_{t-1} is the number of rotifers at the beginning of the experimental time interval

(Δt). *Keratella* numbers were log-transformed to assure the normality of data distribution. The overall growth rate for the whole experimental period (five days) was calculated as a mean of daily growth rates. Egg numbers are presented as cumulative curves, which represent the daily increase in egg numbers, fitted through non-linear regression models using the program Table Curve 2D, version 5.0.

5.2.3 Statistical Analyses

Differences in total population growth rates of *Keratella* were tested for significance using one-way ANOVA followed by the post-hoc Dunnett's *t*-test, which pairwise compares experimental treatments against control treatments. Increases in rotifers' egg numbers in experimental treatments were tested against control treatments using the non-parametric Mann-Whitney *U*-test. Rotifers' growth rates and cumulative egg numbers were correlated with prey absolute and relative amounts of fatty acids, amino acids, and sterols using the non-parametric Spearman rank correlation method in cases where positive growth and egg production of the rotifers were observed. All statistical tests were perfomed using Statistica for Windows, version 5.01 (Stat Soft). A detailed description of the biochemical analyses as well as the biochemical data used in the present study for investigating nutritional quality of heterotrophic protists are provided elsewhere (chapters 2 and 3). For this part we additionally calculated the relative amounts of fatty acids and amino acids, based on the concentration data presented in chapter 2.

5.3 RESULTS

5.3.1 Growth and Reproduction Experiments

Population growth rates of *Keratella* fed algivores (Treatments 1 and 2) and the alga *Cryptomonas* as a sole prey (Treatment 3) were significantly higher than population growth rates of *Keratella* fed bacterivorous protists (Treatments 4 and 5; one-way ANOVA, F=24.01, Dunnetts *t*-test $P<0.001$; Fig. 1). No significant differences were observed when comparing population growth rates of *Keratella* fed bacterivorous protists versus growth rates in the control treatment without prey (one-way ANOVA, F=24.01, Dunnetts *t*-test, all $P>0.05$; Fig. 1).

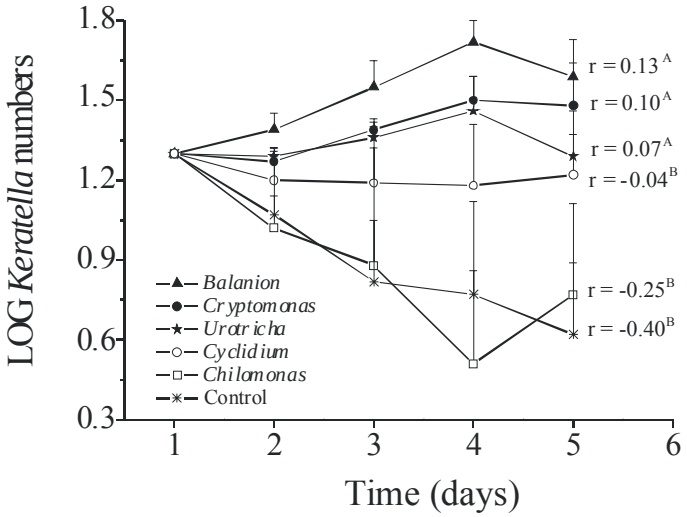

Fig. 1 – Population growth curves of *Keratella quadrata* feeding on the alga *Cryptomonas phaseolus*, the heterotrophic protists, and in the control treatments without prey. Log numbers of *K. quadrata* individuals (average ± upper S.D.) represent a total of at least 5 replicates per treatment. Same letters indicate similar population growth rates (r) of the rotifers when compared to the growth rates obtained in the control treatments with *C. phaseolus* and without prey (one-way ANOVA followed by the Dunnetts *t*-test).

Curves of cumulative egg numbers of *Keratella* were similar for rotifers fed *Balanion* and *Cryptomonas* (Treatments 1 and 3; Mann-Whitney *U*-test, $P>0.05$; Fig. 2). However, the shape of the curves differed slightly, suggesting different daily accumulation rates (see Table 11 for equations).

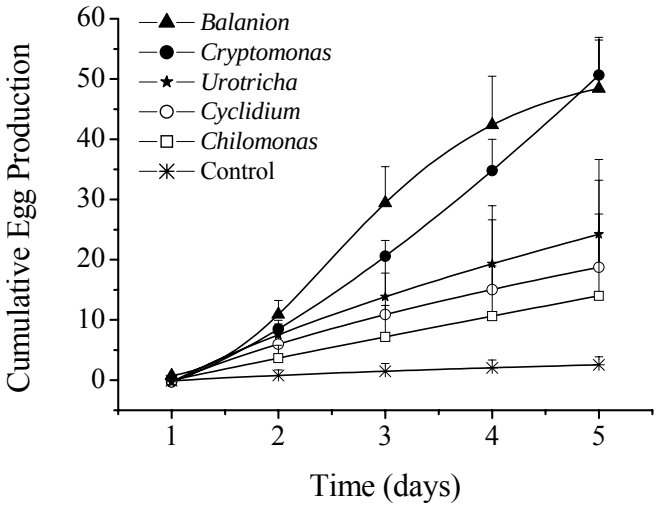

Fig. 2 – Cumulative curves of egg numbers of *Keratella quadrata* feeding on the alga *Cryptomonas phaseolus*, the heterotrophic protists, and in the control treatments without prey. Curves were fitted according to non-linear regression models (see Table 11 for equations). *K. quadrata* cumulative egg numbers (average ± upper S.D.) represent a total of at least 5 replicates per treatment.

A diet of *Urotricha* (Treatment 2) resulted in lower cumulative egg numbers than those observed on a diet of *Cryptomonas* and *Balanion* (Mann-Whitney *U*-test, $P<0.05$), but higher than those observed in the control treatment without prey (Mann-Whitney *U*–test, $P<0.05$). Cumulative egg numbers of *Keratella* in the *Cyclidium* and *Chilomonas* treatments (Treatments 4 and 5) were lower than those observed on a diet of *Balanion* or *Cryptomonas* as sole prey (Treatments 1 and 3; Mann-Whitney *U*-test, $P<0.05$), but they were higher than the cumulative egg numbers observed in the control treatment without prey (Mann-Whitney *U*-test, $P<0.05$).

5.3.2 Relationship between Prey Biochemical Composition and *Keratella*'s Growth and Reproduction

Significant positive correlations were found between cumulative egg numbers and both the absolute concentration and the relative amounts of total polyunsaturated fatty acids (PUFA), γ-linolenic acid (18:3ω6), 20:3ω6, arachidonic acid (20:4ω6), and docosapentaenoic acid (22:5ω3) (Table 12). Significant positive relationships were additionally found for the absolute EPA (20:5ω3) concentrations and the relative amounts of the PUFA 22:2ω6, 22:5ω6, and DHA. Significant negative correlations were observed between *Keratella*'s egg numbers and the absolute concentration and relative amount of linoleic acid (18:2ω6), the absolute concentration of stearidonic acid (18:4ω3), and the relative amount of 22:3ω6 (Table 12).

Desmosterol and ergosterol absolute concentrations and relative amounts as well as stigmastanol relative amounts were positively correlated with the cumulative egg numbers of *Keratella* (Table 13). A significant positive relationship was observed between the relative amounts of the amino acid leucine and the cumulative egg numbers of *Keratella* (Table 13).

Because algivorous ciliates and the alga *Cryptomonas* were the only prey capable of sustaining positive population growth rates of the rotifers throughout the experiments, we performed non-parametric Spearman rank correlation between population growth rates and biochemical composition considering only feeding experiments on algivorous ciliates and *Cryptomonas*. Nevertheless, we did not detect any significant correlation between *Keratella*'s population growth rates and the fatty acid, amino acid, and sterol composition of the algivorous ciliates nor the alga *Cryptomonas* (Spearman Rank correlation, all $P>0.05$).

5.4 DISCUSSION

Interestingly, protist prey species grown on different dietary sources, but showing similar concentration ranges of several biochemical compounds, led to significantly different population growth rates and egg production of *Keratella*. For instance, relative amounts of polyunsaturated fatty acids and absolute sterol concentrations in the bacterivores *Cyclidium* and *Chilomonas* were partially within the same range, or even higher (e.g. cholesterol, sitosterol, and campesterol) than the concentrations found in at least one algivorous species (chapters 2 and 3). However, bacterivores led to negative population growth rates, which did not significantly differ from the growth rates in control treatments without prey (Fig. 1). This finding is in contrast to previous reports on good nutritional quality of bacterivorous protists for daphnids (Sanders and Porter, 1990; Lair and Picard, 2000). On the other hand, inadequate nutritional quality of bacterivorous protists has already been demonstrated for the rotifer *B. calyciflorus* fed the flagellate *C. paramecium* or the ciliate *Tetrahymena pyriformis* Ehrenberg 1830 (Mohr and Adrian, 2002b), and for cladocerans fed the ciliates *Cyclidium glaucoma* Müller, 1773 (Bec et al., 2003) and *Cyclidium* sp. (De Biase et al., 1990). The opposite patterns observed for *Keratella*'s growth rates between feeding experiments with algivores and bacterivores, despite the similar biochemical profiles partially exhibited by algivores and bacterivores, led us to conclude that prey biochemical composition was not constraining *Keratella*'s population growth. When feeding on bacterivores, the biochemical parameters we investigated had obviously no primary influence on rotifer population growth rates, since these were as negative as in the control treatments without prey. Even in cases of positive population growth rates of *Keratella* (feeding on *Balanion*, *Urotricha*, and *Cryptomonas*) we did not observe significant relationships with prey biochemical composition, supporting a possible non-limitation of population growth by biochemical compounds. The alga *Cryptomonas* may have compensated for deficiencies in the algivorous ciliates, since no significant differences were observed among population growth rates of *Keratella* fed either the algivores or *Cryptomonas* only. However, the slight tendency of higher population growth rates of *Keratella* fed *Balanion* compared to *Keratella* fed *Cryptomonas* and *Urotricha*, along with significant higher cumulative egg numbers supported by a diet of *Balanion* than those provided by *Urotricha*, suggests a supplementary effect of *Balanion* to a sole *Cryptomonas* diet. It remains unclear if the supplementation effect of *Balanion* was due to its

biochemical features or to other factors, such as mineral composition, feeding behaviour of the rotifers, or interactions among different food quality aspects.

On the other hand, the significant positive relationship between *Keratella*'s egg numbers and prey biochemical composition suggests a primary influence of prey biochemistry on *Keratella*'s nutrition. For instance, the positive relationship with both the absolute concentrations and the relative amounts of total polyunsaturated fatty acids suggests the importance of those compounds for *Keratella*'s reproduction. Particularly the relationships with essential ω3 polyunsaturated fatty acids, such as EPA (20:5ω3), DPA (21:5ω3), and DHA (22:6ω3) find credence in the literature, as those fatty acids have been found to limit growth and reproduction of a number of planktonic predators, such as daphniids (DeMott and Müller-Navarra, 1997; Von Elert, 2002; Bec et al., 2003) and copepods (Jónasdóttir, 1994). Feeding experiments of *Keratella* on protist cells supplemented with single fatty acid will be necessary to support this finding.

Interestingly, significant positive relationships were detected with ω6 fatty acids, suggesting the importance of those fatty acids for *Keratella*'s reproduction. A limited ability for elongation and desaturation of 18-ω6 precursors such as γ-linolenic acid (18:3ω6) into arachidonic acid (20:4ω6) has been suggested for the rotifer *Brachionus plicatilis* (Lubtzens et al., 1985). It would be interesting to follow the fate of dietary ω6 fatty acids in *Keratella*'s metabolism in order to test the ability for PUFA synthesis in this species and to understand of the role of ω6 PUFA for the rotifer's nutrition. On the other hand, a possible limitation by ω3 fatty acids is suggested indirectly because of significant negative relationships between *Keratella*'s cumulative egg numbers and the absolute and relative amounts of linoleic acid (18:2ω6) and the relative amounts of 22:3ω6. Due to their molecular resemblance, ω3 and ω6 fatty acids may compete for the same bonding sites in membranes (Singer, 1994). Hence, an increase of some ω6 fatty acids and the resultant decrease in the relative amount of their concurrent ω3 fatty acids may lead to a shortfall of active bonded ω3 fatty acids, which then become limiting even though they are available in high absolute concentrations. Since we used living prey organisms instead of artificial diets, complex co-limitation mechanisms involving synergistic and/or antagonistic interactions among biochemical substances may have affected the correlations with single fatty acids.

Sterol limitation of growth and reproduction of zooplankton predators has been found to be mainly caused by dietary cholesterol shortage (Von Elert et al., 2003; Hasset, 2004). Cholesterol plays an important role in membrane stabilization and acts as a precursor in hormone synthesis (Moreau et al., 2002). Cholesterol

deficiency has been associated with decreased growth of copepods (Hasset, 2004) and crustaceans (Von Elert et al., 2003), and with retarded development of crustacean larvae (Teshima, 1991). Using our experimental setup, no direct influence of cholesterol on population growth and reproduction patterns of *Keratella* was detected. However, we did observe a strong positive relationship between *Keratella*'s cumulative egg numbers and the sterol desmosterol. Except for acting as a precursor of cholesterol, no particular biological function has been attributed to desmosterol in invertebrates (Teshima, 1991). The positive relationship found between desmosterol and *Keratella* egg numbers may suggest an important function of desmosterol in this rotifer, perhaps as a precursor of cholesterol. Assuming that cholesterol concentrations were limiting rotifers reproduction, *Keratella* may have converted prey desmosterol to obtain cholesterol. Conversion of desmosterol into cholesterol has been described for a wide range of organisms, including crustaceans (Teshima et al., 1982), molluscs (Knauer et al., 1998), and insects (Ikekawa, 1985).

The significant positive relationship with ergosterol, a sterol with 29 carbon atoms (C-29) only detected in *Cryptomonas*, *Urotricha*, and *Chilomonas* (chapter 3, Tables 5 and 6), suggests its influence on *Keratella*'s reproduction. Ergosterol is a typical sterol of fungi, and its functions have been mainly elucidated in studies on yeast. High dietary ergosterol levels have been associated with enhanced growth and reproduction of crustacean larvae (Kanazawa et al., 1971; Teshima and Kanasawa, 1986), suggesting a possible dealkylation at the carbon C-24 of ergosterol into cholesterol. Teshima (1982) proposed a pathway of dealkylation of C-28 e C-29 sterols via desmosterol to cholesterol in crustaceans. Whether or not ergosterol is converted to cholesterol in *Keratella*, thus supplying prey cholesterol deficiencies is still an open question.

The positive relationship observed between *Keratella* egg numbers and the relative amounts of stigmastanol – the only saturated sterol we found in the studied prey organisms, is rather difficult to explain. It may indicate a limitation originating from increased sterol : stanol ratios. The effect of another phytostanol – sitostanol – on the solubilization of cholesterol was examined under in vitro conditions (Mel'nikov et al., 2004). Free sitostanol was shown to reduce the concentration of cholesterol in artificial diets (micelles) via a dynamic competition mechanism (Mel'nikov et al., 2004). However, since stigmastanol was the only saturated sterol observed in our samples, such a competition mechanism is unlikely in our prey organisms.

Since we found only minor differences in the concentrations of amino acids among prey organisms (i.e. those in tryptophan and leucine in *Cryptomonas* and *Balanion*, respectively), we did not expect to find significant associations between prey amino acid and *Keratella* life history parameters. Top predators, such as the rotifer *Brachionus plicatilis* Müller 1786, showed rather constant amino acid composition when fed different algal species or the yeast *Saccharomyces cerevisae* Meyen and Hansen, 1883 (Frolov et al., 1991). Guisande et al. (1999) concluded that the selective retention of amino acids in *Euterpina acutifrons* Dana, 1848 was based on a chemical homeostasis of essential amino acids in this copepod. In our study we found a positive correlation between the cumulative egg numbers of *Keratella* and leucine relative amounts. Leucine has been found to be an essential amino acid involved in egg production and larval development in many species including fish (Fyhn, 1989; Dayal et al., 2003), crustacean (Reddy, 2000), copepods (Laabir et al., 1999), and insects (Chang, 2004).

The fact that biochemical compounds were not correlated with population growth rates but with cumulative egg numbers suggests that biochemical requirements for population growth differ from those for egg production, in the case of *Keratella* fed heterotrophic protists. This is in accordance with the stoichiometric theory that states that growth, reproduction, and survivorship have different metabolic demands (Sterner and Hessen, 1994). In our study, the observed effects of biochemical compounds were related to *Keratella* reproduction patterns, measured as the cumulative numbers of produced eggs. Effects of fatty acid and sterol may be related to egg production and maintenance until hatching. Increased egg production of the copepod *Acartia tonsa* Dana, 1848 was found to be positively influenced by the ω3 fatty acid composition of its algal diet (Jónasdóttir, 1994). Teshima et al. (1982) showed that cholesterol is indispensable for the metamorphosis from nauplii to post-larvae and the survival of the larval prawn *Panulirus japonica* Von Siebold, 1824.

Although algivorous and bacterivorous protists ensured partially similar biochemical composition (chapters 2 and 3), only feeding on algivores resulted in positive population growth rates of *Keratella*. In this case, the differences in nutritional quality of algivores versus bacterivores could not be explained by the investigated biochemical compounds. Our results suggest that single biochemical factors become limiting only under non-limitation by another factor. This is along the lines of the Liebig's Law of the Minimum (Von Liebig, 1855) that states: an organism will become limited by whatever resource that is in lowest supplies compared to the organism's needs. In a recent study, Becker and Boersma (2003) showed that limitation effects of an essential fatty acid on *Daphnia* life history

could only be detected below a specific prey nutrient threshold. Above this threshold, the daphniids were limited by nutrient ratios. It seems that, depending on the development stage of the daphniids, mineral limitation may occupy a higher position than biochemical compounds do in limiting daphniid nutrition. However, such limitation effects are more apparent in studies in which limitation of combined mineral and biochemical components is artificially induced, generally by supplementing prey organisms with one component in detriment to another. Unfortunately, despite the undoubted importance of those experimental studies, such extreme limitation conditions may not necessarily correspond to natural conditions. In nature, predator nutrient deficiencies resulting from ingesting a low quality prey may be compensated for by other strategies, like mixed ingestion of different prey. Here, we found support for biohchemical limitation in a trophic relationship between a rotifer species and different heterotrophic protist prey, whose original biochemical or mineral composition were not previously modified or artificially supplemented. Our data suggest a primary role of protist biochemistry for *Keratella* reproduction but only a secondary role for *Keratella* population growth, although we could not identify the primary limitation factors in this later case.

Interestingly, biochemical limitation effects were only evident for *Keratella*'s reproduction but not for population growth. In nutritional quality studies, whereby prey composition is kept unaltered, the necessity of considering synergetic and/or antagonistic aspects of simultaneous mineral and biochemical limitation for predators becomes clear, and may have been responsible for the absence of significant correlations between *Keratella*'s population growth and prey biochemistry in our study. Further, the influence of non-quantified chemicals, like vitamins and trace-elements, and especially of synergetic and/or antagonistic interactions among biochemical compounds cannot be excluded.

In a broader ecological sense, our study emphasizes heterotrophic protists as an alternative source of essential substances for mesozooplankton predators and an important link between microbial and classic food-webs. However, requirements of the predator may be not completely supplied by feeding on bacterivorous protists, as evidenced by the negative population growth rates of *Keratella* fed *Chilomonas* or *Cyclidium*.

6. A METHODOLOGICAL TEST FOR SUPPLEMENTING *CHILOMONAS PARAMECIUM* WITH ESSENTIAL FATTY ACIDS AND ITS APPLICATION FOR EVALUATING NUTRITIONAL QUALITY FOR *KERATELLA QUADRATA*

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Keywords: Fatty acids; Supplementation; Heterotrophic protists; Nutritional quality; Rotifers

ABSTRACT

A straightforward method to determine whether a single dietary biochemical compound is limiting for a predator is to use dietary supplements. We tested whether a fatty acid supplementation technique using bovine serum albumin as a carrier, previously developed for autotrophic protists, is also appropriate for fatty acid supplementation of *Chilomonas paramecium* – a flagellated heterotrophic protist. *C. paramecium* was successfully enriched with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both known to be essential fatty acids for crustacean zooplankton. Standardized feeding experiments were performed to test the nutritional value of EPA and DHA for the rotifer *Keratella quadrata* by following growth rates and egg numbers of the rotifer on a diet of supplemented and non-supplemented *C. paramecium*. The results were compared to the performance of the rotifer fed *Cryptomonas phaseolus*, an alga known to support growth and reproduction of *Keratella*. Preparing *C. paramecium* enriched with EPA and DHA in the same concentration range as found in *C. phaseolus* allowed us to test the direct nutritional effects of the two fatty acids for *K. quadrata*, qualitatively and quantitatively. Growth rates and egg production of *K. quadrata* were highest when fed the alga *C. phaseolus*. Among the different supplementation treatments with *C. paramecium*, egg development of *K. quadrata* was significantly enhanced on a diet of *C. paramecium* enriched with DHA, whereas no significant effects could be attributed to EPA enrichment. Thus, factors other than EPA or DHA limit food quality of *C. paramecium* for *K. quadrata*.

6.1 INTRODUCTION

Heterotrophic protists such as ciliates and heterotrophic nanoflagellates occupy an intermediate position in aquatic food webs, linking bacterial production to the energy flow into the classical food web (Pomeroy 1974; Azam et al. 1983), as they are themselves preyed upon by the mesozooplankton such as crustaceans (Klein Breteler, 1980; Stoecker and Egloff, 1987; Klein Breteler et al., 1999) and rotifers (Mohr and Adrian, 2001, 2002a,b; chapter 5). Despite their important function as trophic linking, information on the quality of heterotrophic protists for mesozooplankton predators is rather limited to date (but see chapter 5).

Information on chemical compounds which confer prey nutritional quality mainly derived from correlative evidence relating biochemical composition such as fatty acids or the elemental stoichiometry of algae and corresponding growth rates of hervivorous zooplankton – mainly daphnids. Highly unsaturated fatty acids (HUFA) such as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) have been identified, among others, as being important in limiting growth and reproduction of freshwater zooplankton (Müller-Navarra, 1995; Wacker and Von Elert, 2001; Müller-Navarra et al., 2004). An elegant way to verify correlative evidence is to assess the role of individual HUFA directly by supplementing a diet artificially followed by the evaluation of the supplementation effects on the performance of a predator. Current supplementation techniques involve the addition of lipid microcapsules or liposomes (Ravet et al., 2003) and lipid emulsions (DeMott and Müller-Navarra, 1997; Boersma et al., 2001; Park et al., 2003) to an algal prey. Unfortunately, these techniques frequently used mixtures of HUFA, so that effects of a single fatty acid could not be identified. A new technique recently proposed by Von Elert (2002) allows the manipulation of algal fatty acid profiles by the addition of a single fatty acid, which is directly assimilated by algal cells using bovine serum albumin as a carrier. The use of bovine serum albumin prevents toxic effects of free fatty acids. Furthermore, the fatty acid is offered to the predator directly as part of its diet, by which problems related to inefficient ingestion or assimilation are avoided.

In previous feeding experiments with the rotifer *Keratella quadrata* offered a number of different protist prey species we found correlative evidence that EPA and DHA do also promote egg production of this rotifer (chapter 5). Given our interest in the biochemical nature of protist nutritional quality we tested whether the technique developed for autotrophs (Von Elert, 2002) is also applicable for heterotrophic protists. We chose the flagellate *Chilomonas paramecium*, which

was supplemented with EPA and DHA. By choosing this flagellate we expected pronounced supplemental effects because *C. paramecium* comprised the lowest natural EPA concentrations out of four examined heterotrophic protists (chapter 2) as well as lower DHA concentrations as compared to the autotrophic flagellate *Cryptomonas phaseolus* (chapter 2). Our intention was to enhance EPA and DHA concentrations of *C. paramecium* up to the natural concentrations observed in *C. phaseolus*, which is known as a good quality alga. In standardized feeding experiments we then tested the effects of EPA and DHA supplementation on growth rates and egg production of *K. quadrata*. Our study contributes to the overall controversial discussion about the role of EPA and DHA in zooplankton performance.

6.2 METHODOLOGY

6.2.1 Cultures

The flagellate *Chilomonas paramecium* (403 ± 288 μm^3, mean biovolume ± SD) was cultured in Volvic water at 18 ± 1°C under a 16:8 h light : dark regime, and fed bacteria grown on previously autoclaved polished rice corns. The cryptomonad *Cryptomonas phaseolus* (392 ± 125 μm^3) was obtained from the Algal Culture Collection of the University of Göttingen, Germany, and cultured in WC medium (Guillard and Lorenzen, 1972) at 17 ± 1°C under a 16:8 h light : dark regime. Algal cultures were non axenic, but bacteria accounted for less than 2 % of the total organic carbon contents in the cultures. The rotifer *Keratella quadrata* was originally isolated from Müggelsee, an eutrophic lake located in Berlin, Germany. Rotifers were cultured in WC medium in weekly diluted batch cultures and fed *C. phaseolus* (1.5 mgC L^{-1}). Cultures were kept under the same temperature and light : dark regimes applied for *C. phaseolus*. Henceforth, species are referred to by their genus names.

6.2.2 Supplementation with Essential Fatty Acids

Chilomonas was enriched with EPA and DHA following the protocol given in Von Elert (2002). *Chilomonas* cultures were incubated with EPA (Sigma E2011, purity ≥99%) and DHA (Sigma D2534, purity ≥98%) along a concentration range between 70 and 360 µg mgC^{-1} of EPA or DHA in the incubation medium. An ethanolic solution containing each single fatty acid was added to a bovine serum

albumin solution (BSA, Sigma A7906, 4 mg mL^{-1}). Subsequently, 10 mL WC medium and a minimum *Chilomonas*' biomass of 4 mgC were added to the solution. *Chilomonas* were previously separated from their culture medium by filtration through a 10 μm silk net and resuspended in Volvic water. Resuspended cells were concentrated to 4 mgC by repeated centrifugation (3000 rpm for 5 min). The resulting suspensions (fatty acids + BSA + WC medium + 4 mgC *Chilomonas* biomass) were incubated under rotation (100 rpm) at 18°C for 4 h in the light (100 μmol m^{-2} s^{-1}). After incubation, cells were repeatedly rinsed with WC medium in order to remove excess BSA and free fatty acids, and then collected on pre-incinerated GF/C glass fibre filters (Whatman) and stored at –20°C until analysis. All treatments were run in five replicates from at least three different *Chilomonas* batches (n=15 – 20). The integrity of *Chilomonas* cells was checked by standard microscopy. Control treatments were incubated without fatty acids (non-supplemented *Chilomonas*). An additional treatment consisting of *Chilomonas* incubated with BSA only- was carried out in order to eliminate the influence of essential amino acids on *Keratella*'s performance.

6.2.3 Fatty Acid Analysis

Filters containing material for analysis were extracted in a 2:1 v/v chloroform-methanol solution at 20°C for 4 h (Folch et al., 1957). An internal standard was added to the samples (tricosanoic acid, 0.2 mg mL^{-1}). Fatty acid methyl-esters (FAME) were formed by addition of a methanolic sulphuric acid solution (5% v/v) and heating the samples for 4 h at 80°C (Weiler, 2001). An aliquot of 0.2 μL of the samples was injected into a Varian Star 3600 CX series gas chromatograph, equipped with a fused silica capillary column (Omegawax 320, SUPELCO, 30 m x 0.32 mm). An initial temperature of 180°C (2 min) was applied, subsequently heated at 2°C min^{-1} to 200°C, which was held isothermally for 33 min. Injector and FID detector temperatures were 250°C and 260°C, respectively. Helium was used as a carrier gas. FAMEs identification were made by comparing the retention times with retention times of a standard solution (Supelco FAME Mix 47885–4, PUFA Nr. 3–47085–4 and PUFA Nr. 1–47033) and values were expressed as concentrations per carbon biomass of *Chilomonas*. The concentrations of EPA and DHA are given for samples incubated with EPA, DHA, BSA only, and in non-supplemented samples as well.

6.2.4 Feeding Experiments with *Keratella quadrata*

We tested the importance of EPA and DHA by following growth rates and egg production of *Keratella* in prey treatments with supplemented and non-

supplemented *Chilomonas*. The rotifers were separated from their algal food and resuspended in Volvic water 12 hours prior to the start of the experiments. Twenty *Keratella* without eggs were placed into 15 mL chambers in macrotiter plates containing the different diets, offered in concentrations above the incipient limiting level, previously determined in functional experiments (1 mgC L^{-1}, Boëchat unpublished data). The rotifers received fresh prey suspensions daily over a 5 day experimental period. As a common practice in such feeding experiments, the predators are daily transferred into a new chamber with fresh prey suspension. However, due to the high fragility of *Keratella* to manual handling, we decided not to remove them from the experimental chambers. Instead, we added fresh prey suspensions to the chambers, starting the experiments with an initial volume of 4 mL and adjusting the prey densities daily to a final volume of 12 mL at day 5. Prey treatments were as follows: *Chilomonas* previously supplemented with EPA (*Chilomonas* + EPA) and DHA (*Chilomonas* + DHA), BSA only (*Chilomonas* + BSA), non-supplemented *Chilomonas* (*Chilomonas*), and a control treatment without food (no food). A diet of non-supplemented *Cryptomonas* was used as a reference, since *Cryptomonas* is known to support growth and reproduction of *Keratella* (chapter 5). Incubation concentrations of EPA and DHA were 90 μg mgC^{-1}, which resulted in an increase in EPA (13.3 μg EPA mgC^{-1}) and DHA (33.6 μg DHA mgC^{-1}) concentrations in *Chilomonas* equivalent to the range found in *Cryptomonas* (11.2 μg EPA mgC^{-1} and 40.2 μg DHA mgC^{-1}; see chapter 2). This allowed us to directly test the nutritional effects of EPA and DHA for *Keratella*. Macrotiter plates containing each treatment in five replicates were incubated at 18 ± 1°C under a 16:8 h light : dark regime. Rotifers and eggs were enumerated at x20 magnification. Population growth rates (r) of the rotifers were calculated assuming exponential growth according to:

$$r = \frac{\ln(N_t) - \ln(N_{t-1})}{\Delta t}$$

Where N_t is the number of rotifers at the end of the experimental time interval (Δt) and N_{t-1} is the number of rotifers at the beginning of the experimental time interval (Δt). *Keratella*'s numbers were log-transformed to assure normality of data distribution. The overall growth rate for the entire experimental period (five days) was calculated as a mean of daily growth rates. Egg numbers are presented as cumulative curves, which represent the daily increase in egg numbers, fitted by non-linear regression models using the program Table Curve 2D, version 5.0.

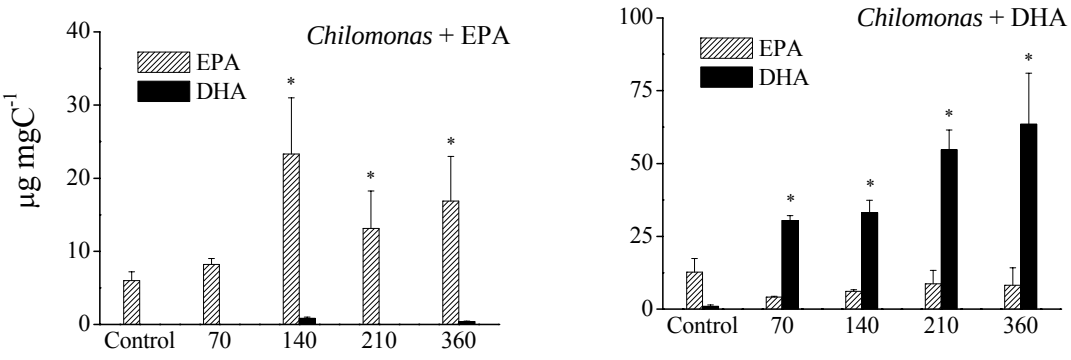
6.2.5 Statistical Analysis

Differences in the percentages of each fatty acid (EPA or DHA) in the total fatty acid profile of supplemented versus non-supplemented *Chilomonas* were tested for significances using one-way ANOVA followed by the Dunnett *t*-test, which pairwise compared supplementation treatments against non-supplemented treatments (Statistica for Windows, version 5.01, Stat Soft). The same statistical procedure was applied for testing differences in total population growth rates and cumulative egg production of *Keratella* among feeding treatments.

6.3 RESULTS

6.3.1 EPA and DHA Supplementation of *Chilomonas paramecium*

The natural concentration of EPA in *Chilomonas* was 5.9 ± 1.2 µg mgC^{-1} (Fig. 1). EPA concentrations were significantly elevated in EPA supplemented *Chilomonas* (23.3 ± 7.7 µg mgC^{-1}) as compared to non supplemented *Chilomonas* (ANOVA $F=10.73$, $P<0.01$, Dunnett *t*-Test, $P<0.05$; Fig. 1). Beyond an incubation concentration of 140 µg EPA mgC^{-1}, EPA uptake by *Chilomonas* reached saturation (Fig. 1).



Fatty acid concentration range in incubation medium (µg mgC^{-1})

Fig. 1 – Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) concentrations in *Chilomonas paramecium* supplemented with different incubation concentrations of either EPA or DHA versus concentrations in non-supplemented *Chilomonas* (control). Significant differences between supplemented and non-supplemented (control) treatments are indicated by asterisks (ANOVA followed by Dunnett *t*-Test).

The natural DHA concentration in *Chilomonas* was 0.94 ± 0.53 µg mgC^{-1} (Fig. 1). DHA concentrations in supplemented *Chilomonas* increased with increased DHA concentrations in the incubation medium and were significantly elevated to up to 63.5 ± 17.5 µg mgC^{-1} (ANOVA F=10.73, P<0.01, Dunnett t-Test, P<0.01; Fig. 1) as compared to non-supplemented *Chilomonas*. No significant changes were observed in DHA concentrations when supplementing *Chilomonas* with EPA (ANOVA F=0.85, P=0.51) and vice-versa (ANOVA F=3.09, P=0.08). Microscopy revealed no cell damage of supplemented *Chilomonas*.

6.3.2 Feeding Experiments with *Keratella*

Keratella fed *Cryptomonas* exhibited higher population growth rates (ANOVA F=14.74, P<0.01) and cumulative egg numbers (ANOVA F=3.25, P<0.01; Fig. 2) than rotifers fed supplemented *Chilomonas*.

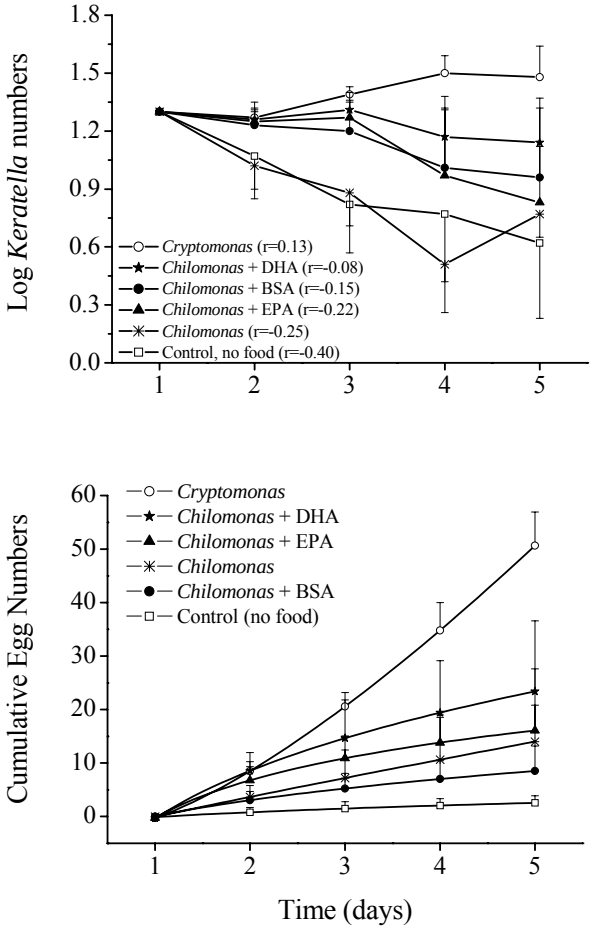


Fig. 2 – *Keratella quadrata* log-numbers and population growth rates (upper panel) and cumulative egg numbers (lower panel). Treatments were: rotifers fed non-supplemented *Cryptomonas phaseolus* (*Cryptomonas*), *Chilomonas paramecium* supplemented with docosahexaenoic acid (*Chilomonas* + DHA), eicosapentaenoic acid (*Chilomonas* + EPA), with bovine serum albumin without fatty acids (*Chilomonas* + BSA) as well as non-supplemented *Chilomonas paramecium* (*Chilomonas*), and a control treatment without food (no food).

Feeding on *Chilomonas* supplemented with DHA improved rotifers' population growth rates and reproduction based on egg numbers (Fig. 2), when compared to non-supplemented *Chilomonas* (Dunnett *t*-Test, $P<0.05$) or controls without food (Dunnett *t*-Test, $P<0.01$). Although we found a slight tendency of higher population growth rates and cumulative egg numbers on a daily time scale in EPA supplemented treatments, EPA enriched *Chilomonas* did not significantly improve population growth rates of the rotifer, when compared to non-supplemented *Chilomonas* (Dunnett *t*-Test, $P=0.97$) or to the control treatments without food (Dunnett *t*-Test, $P=0.09$). Egg numbers were unaffected by EPA supplementation (Dunnett *t*-Test, $P=0.69$). In the *Chilomonas* + BSA treatments population growth rates of *Keratella* were higher than those found in non-supplemented *Chilomonas* or the control treatments without food (Dunnett *t*-Test, $P<0.05$). Egg numbers remained unaffected in the *Chilomonas* + BSA treatments (Dunnett *t*-Test, $P=0.35$).

6.4 DISCUSSION

The fatty acid supplementation technique proposed by Von Elert (2002) for autotrophic protists proved to be equally appropriate for supplementing *Chilomonas* with EPA and DHA. The concentration dependent relationship between fatty acid concentrations in the incubation medium and fatty acid uptake by *Chilomonas* enabled us to prepare prey organisms of defined fatty acid contents. This generally allows the evaluation of direct effect of dietary specific fatty acids on zooplankton performance, qualitatively and quantitatively. As no significant changes were observed in the EPA concentrations in DHA supplemented *Chilomonas* and vice-versa, interconversion of those molecules, which may have complicated the interpretation of single EPA and DHA effects on *Keratella*'s performance, could be excluded. Although cellular EPA and DHA concentrations of *Chilomonas* had been elevated to concentrations found in *Cryptomonas*, *Chilomonas* did not support rotifers' growth and high egg numbers as *Cryptomonas* did. This indicates that EPA and DHA were not the major factors limiting the performance of *Keratella* fed *Chilomonas*. Since both *Cryptomonas* and *Chilomonas* are morphologically very similar (similar size and shape, both flagellated cryptomonads) differences in the trophic mode may relate to the differences in their nutritional quality.

Nevertheless, DHA supplementation of *Chilomonas* significantly improved its nutritional quality as prey for *Keratella*, when compared to non-supplemented *Chilomonas*. This suggests that DHA was present in limiting concentrations in non-supplemented *Chilomonas*. This is supported by the positive correlation between *Keratella*'s egg numbers and DHA dietary concentrations derived from laboratory experiments (chapter 5). Especially reproduction of *Keratella* seems to be positively affected by DHA concentrations. Our results observed for *Keratella* are consistent with findings found for other zooplankton organisms. Dietary DHA, together with EPA and other long chained fatty acids, has been found in high amounts in copepod's eggs (Sargent and Falk-Petersen, 1988). Moreover, in some crustaceans EPA and DHA have been found in 2 – 5 fold higher amounts in eggs and ovaries Than in other female tissues (Hayashi, 1976), suggesting the importance of these fatty acids for embryonic survival and growth. DHA is also believed to play a significant role during larval development and metamorphosis of marine molluscs (Delaunay et al., 1993) and to promote enhanced larval growth of the zebra mussel *Dreissena polymorpha* (Wacker et al., 2002). The fact that DHA supplementation lead to increased egg numbers but only slightly enhanced *Keratella* population growth rates suggests that reproduction and population growth have different metabolic demands, which is in accordance with the stoichiometric theory (Sterner and Hessen, 1994). Supplementation of *Chilomonas* with only BSA improved population growth of the rotifers when compared to non-supplemented cells or to the control treatments without food, suggesting that *Chilomonas* may be short in amino acid concentrations. Amino acid limitation has indeed been shown to constrain egg production and hatching success of copepods (Kleppel et al., 1998; Guisande et al., 2000).

There is a debate about which HUFA of the ω3 class – such as EPA or DHA – primary limits zooplankton nutrition. Although field studies have shown a significant correlation between sestonic EPA concentrations and growth rates of daphnids (Müller-Navarra, 1995), supplementation of an algal diet with EPA did not improve growth and reproduction of *Daphnia magna* (Von Elert and Wolffrom, 2001). In a study combining EPA supplementation and mineral limitation, Becker and Boersma (2003) showed that EPA limiting effects on growth rates of *Daphnia magna* were only detectable below a certain nutrient ratio (C:P = 350) in the algal diet. Other studies, in contrast, showed evidence for the positive effects of DHA supplementation for the zebra mussel *Dreissena polymorpha* (Wacker et al., 2002) and for the calanoid *Acartia tonsa* (Kleppel et al., 1998). Our study adds to the controversial discussion about the role of HUFA in zooplankton nutrition and suggests DHA limitation at the interface

heterotrophic protists – zooplankton. However, DHA supplementation could not completely compensate the low nutritional quality of *Chilomonas* for *Keratella*, suggesting the role of other limitation sources.

Although it has been generally accepted that protists are an important component linking the microbial to the classical food web, virtually nothing is known about their biochemical composition and nutritional quality. In this thesis, I examined (1) whether the biochemical composition of free-living protists depends on their dietary sources, (2) their trophic mode and (3) whether their biochemical composition determines their nutritional quality for zooplankton predators. In this context, four hypotheses were tested (see *Outline of the Thesis*). In the following, I refer to each hypothesis and give an overview of the most important findings and implications for the nutritional quality of protists. Finally, I summarize both aspects – biochemical composition and nutritional quality of protists – in an ecological context.

Hypothesis 1. The biochemical composition of heterotrophic protists depends on the biochemical composition of their diet.

To test this hypothesis I analysed the fatty acid and amino acid (chapter 2) as well as the sterol composition (chapter 3) of two protist species grown on an algal diet and two species grown on a bacterial diet. Given known differences in the biochemical composition between algae and bacteria (Harvey et al., 1997), I expected to find these differences reflected in the composition of algivorous and bacterivorous protists. By comparing two species grown on the same diet, I discussed species-specific differences in the biochemical metabolism of those heterotrophic protists.

The most important finding here was that the fatty acid, amino acid, and sterol composition of the studied protists generally reflected the composition of their diet, but absolute concentrations were often higher in the protists than in their diet (you are not what you eat!). Higher concentrations in the heterotrophic protists as compared to dietary concentrations suggest that accumulation of biochemical compounds takes place. Goulden and Place (1990) showed that adult daphniids accumulate lipids preferentially, relative to other biochemical components of their diet. To explain the accumulation of fatty acids, sterols, and amino acids in the here studied heterotrophic protists, as well as of lipids in the daphniids, one must assume that these organisms (1) preferentially assimilate lipids and amino acids,

(2) preferentially metabolize assimilated carbohydrates while storing lipids and amino acids, or (3) synthesize lipids and amino acids.

Efficient assimilation may have been related to high ingestion rates. An estimate of the ingestion rates needed to reach the EPA concentrations we measured in the protists, versus known published ingestion rates for *Balanion planctonicum* and *Cyclidium* sp. (Müller and Schlegel, 1999; Šimek et al., 1994) makes this a reasonable assumption. However, similar carbon-specific concentrations in organisms of different size, like *Cyclidium* and *Balanion*, may not only rely on high ingestion rates. Preferential assimilation of fatty acids, sterols, and amino acids, or preferential metabolism of carbohydrates or other carbon compounds may have contributed to the accumulation of biochemical compounds in the studied heterotrophic protists. Carbohydrates are not as energetic as lipid or protein molecules (Stryer, 1995), but carbohydrate metabolism may have provided the protists with enough energy for basic processes such as growth and survival. In this case, the differential allocation of lipids and amino acids for cellular functions other than energy production would explain the accumulation of those biochemicals in the heterotrophic protists. These may possibly have stored biochemical compounds for building structural molecules, such as phospholipids, as suggested by the positive correlation between cell size and cellular fatty acid concentrations (chapter 2).

Synthesis of biochemical compounds such as fatty acids and sterols by protists has been already described (Koroly and Connor, 1976; Klein Breteler et al., 1999). First evidences for the synthesis of highly unsaturated fatty acids, like EPA and DHA, and also sterols by the heterotrophic dinoflagellate *Oxyrrhis marina* (Klein Breteler et al., 1999) suggest to reconsider the assumption that heterotrophic protists are unable to synthesize complex lipid molecules. For instance, because DHA was not observed in the bacterial diet of *Chilomonas*, the only explanation for the presence of DHA in this flagellate should be synthesis, probably through elongation of linolenic acid (see Appendix 1). However, synthesis of unsaturated fatty acids may be inefficient in phagotrophic protists. *Cryptomonas* and *Chilomonas* are both cryptomonads, and they both contained DHA. However, DHA concentrations were higher in *Cryptomonas* than in *Chilomonas*. Possibly, DHA synthesis in *Chilomonas* is not as efficient as in *Cryptomonas*, which could be due to species-specific differences in fatty acid metabolism or to the trophic mode of the organisms, since *Cryptomonas* grown as autotroph and *Chilomonas* as heterotroph.

I found evidence for sterol synthesis by heterotrophic protists in my study, especially concerning the phytosterols *ergosterol* and *brassicasterol*. Both sterols were found in *Chilomonas*, although not present in their bacterial diet. *De novo* synthesis of sterols has been described for some amoebae species belonging to the genera *Acanthamoeba* and *Naegleria* (Raederstorff and Rohmer, 1987a,b). In those species, ergosterol was the predominant sterol, with cycloartenol serving as a precursor. Cycloartenol was also found in the here studied protist species, including *Chilomonas*. This finding also points to ergosterol synthesis in *Chilomonas*.

The presence of high phytosterol concentrations, especially stigmasterol, instead of cholesterol in all analyzed protists suggests that phytosterols may have analogous regulatory functions as cholesterol in cell membranes of these species. Cholesterol was only found in minor amounts in the protists, except for *Cyclidium*, which also contained the triterpenoids tetrahymanol, diplopterol, and diploptene, which are typically found in ciliates and some bacteria (Ourisson et al., 1987). In *Tetrahymena*, synthesis of tetrahymanol was shown to be hampered by exogenous supply of stigmasterol, suggesting that synthesis is regulated by energetic trade-off mechanisms or possibly through molecular concurrence between these triterpenoids for the same enzymatic bonding sites (Holler et al., 1993).

Conclusion: The answer to the question "Does the biochemical composition of free-living protists depend on their dietary sources?" is: "Yes, but not exclusively". Dietary composition influences protist composition, but the studied species were able to accumulate dietary fatty acids, sterols, and amino acids. They could have been able to differentially allocate lipids and proteins for structural functions and to preferentially metabolise carbohydrates for generating energy. Moreover, synthesis of some compounds is likely, as important precursors of fatty acids and sterols were observed in the heterotrophic protists. The precursors may have been obtained from the diet, but the enzymatic accessories necessary for elongating fatty acids or cycling sterols must come from the protists themselves, as enzyme synthesis and activation is genetically determined. In other words: heterotrophic protists probably get the biochemical composition of their diet, but they are able to modify dietary compounds and to allocate them differentially. Moreover, these metabolic features seem to vary species-specifically, as suggested by the different biochemical composition in two species fed the same diet (e.g. *Chilomonas* had DHA but *Cyclidium* had not).

Future Perspectives: It would be very interesting to follow the fate of dietary compounds and differential allocation of biochemicals in heterotrophic protists. For these purposes experiments involving labelled target molecules which are followed throughout metabolic pathways (time series experiments) in the protist cells are necessary. Information provided by such experiments would contribute to the understanding of metabolic pathways in heterotrophic protists as well as the energetic trade-offs – allocation into structural molecules or energy reserves – which certainly influences the nutritional quality of heterotrophic protists, besides the biochemical composition itself.

Hypothesis 2. The biochemical composition of protists depends on the trophic mode.

In a second approach (chapter 4), I analysed the fatty acid and sterol composition of a single species – the flagellate *Ochromonas* sp. – cultured under three different trophic modes: autotrophic, mixotrophic, and heterotrophic. The aim here was to test the influence of the trophic mode on the biochemical profile of *Ochromonas* sp. and to get some insights on metabolic features related to synthesis and allocation of lipids in this flagellate.

The hypothesis was corroborated: the trophic mode determined the metabolic pathways of *Ochromonas* sp., resulting in different biochemical composition of the flagellates cultured under different trophic modes. Decreasing concentrations of polyunsaturated fatty acids were observed as the trophic mode changed from autotrophic via mixotrophic to heterotrophic growth. Saturated fatty acids were the most abundant fatty acid class in heterotrophs, while polyunsaturated fatty acids were most abundant in autotrophs. Interestingly, the highest concentration of total saturated fatty acids was observed in mixotrophs, suggesting ingestion of bacteria by mixotrophs. Nevertheless, mixotrophs showed similar total concentrations of monounsaturated fatty acids to those found in autotrophs. Moreover, total polyunsaturated fatty acid concentrations in mixotrophs were higher than in heterotrophs, but not as high as in autotrophs. Mixotrophs clearly synthesized some biochemical compounds, which were not present in their bacterial diet. Nevertheless a number of their biochemical compounds were equivalent to those found in the bacteria.

There is an ongoing debate on the meaning of mixotrophy; i.e. the question as to why flagellates, capable of photosynthesis, graze bacteria. Some authors argue

that mixotrophy is a mean of acquiring nutrients for photosynthesis during periods of limitation (Nygaard and Tobiesen, 1993). Other authors suggest that the prime purpose of mixotrophy is to obtain carbon to support growth (Bird and Kalff, 1986; Caron et al., 1990; Jones et al., 1993). If so, carbon obtained by phagotrophy may be primarily used for building structural biomolecules such as carbohydrates and phospholipids, whereas photosynthesis may be responsible for generating substrates for the synthesis of more complex energetic biomolecules, such as triglycerides.

The patterns observed for fatty acids suggest that fatty acid metabolism may be strongly influenced by the trophic mode and by the processes of energy supply – photosynthesis or phagotrophy. In superior plants, the enzyme acetyl CoA carboxylase catalyses the first step of fatty acid synthesis and its activity depends on both light and the rate of fatty acid synthesis in the chloroplasts (Post-Beitenmiller et al., 1992). *De novo* synthesis of lipids in plants also depends on NADPH generated in the light reactions of photosynthesis. Hence, it is expected that rates of lipid synthesis and the relative allocation of photosynthates into lipid synthesis both tend to increase with incubation irradiance (Wainman and Lean, 1992). My findings of an increasing tendency of fatty acid concentrations and degree of unsaturation from heterotrophs to autotrophs are consistent with this tendency of high lipid synthesis rates in the presence of light, if all other conditions remain unaltered.

The sterol pattern found for *Ochromonas* in my study corroborates findings of sterols in other *Ochromonas* species (Halevy et al., 1966; Avivi et al., 1967; Goodwin, 1974), where stigmasterol was also the most abundant sterol present. The sterol metabolism in *Ochromonas* seems to be less dependent on the trophic mode than fatty acids do, as autotrophs and heterotrophs contained similar concentrations of all sterols present. Mixotrophs, on the other hand, showed the lowest sterol concentrations among flagellates, although the relative amounts remained similar to those found in autotrophs and mixotrophs. The high metabolic costs of a mixotroph to run both an autotrophic and a phagotrophic metabolism (Rothhaupt, 1996a) may limit the synthesis rate of some sterols. In mixotrophic *Ochromonas*, sterol synthesis may have been mostly directed to stigmasterol synthesis, as stigmasterol concentrations in mixotrophs were similar to those fond in autotrophs and heterotrophs. It would be interesting to investigate the function of stigmasterol in *Ochromonas*, since stigmasterol synthesis seems to be maintained even at low synthesis rates of other sterols.

The fact that heterotrophs contained higher concentrations of sterols than mixotrophs may relate to the phagotrophic mode of heterotrophs. The metabolic processes discussed in the past chapters for fatty acids and amino acids, including high ingestion rates, preferential metabolism of carbohydrates, and self synthesis, could also be claimed to explain the higher sterol concentrations in heterotrophs than in their bacterial diet. The fact that these metabolic processes are energy-consuming might explain why mixotrophs did not contain sterol concentrations as high as heterotrophs did.

Sterols are less appropriate to differentiate *Ochromonas* sp. by its different trophic modes, as no significant differences were observed between autotrophs and heterotrophs. Fatty acid composition, by contrary, seems to be a very efficient marker of the trophic mode, probably because fatty acid metabolism may be influenced more strongly by the mechanisms of energy uptake: photosynthesis or phagotrophy. Additionally, fatty acids and sterols are expected to respond differently to environmental factors such as light and nutrients, because these lipid classes exhibit differences in their functions and biosynthetic pathways (Parish and Wangersky, 1987; Smith and D'Souza, 1993).

Conclusion: The question "Does the biochemical composition of protists depend on their trophic mode?" could clearly be answered with "Yes, it does" for *Ochromonas* sp. This became especially evident for fatty acids, which differed significantly among *Ochromonas* sp. grown as autotroph, mixotroph, and heterotroph. For sterols, the differences observed between trophic modes seemed to be caused by energetic trade-offs as mixotrophs, living more energy-consuming, had lower sterol concentrations than specialized autotrophs or heterotrophs.

Future Perspectives: It would be interesting to investigate under which set of conditions lipids, especially fatty acids, are allocated into structural (phospholipids in the membranes) or energy reserve molecules (triglycerides) in *Ochromonas* of different trophic modes. Analyses of fatty acid contents in triglycerides and phospholipids would be an adequate method to test for differential lipid allocation. Differential allocation of lipids may affect cellular division rates, and consequently, population growth. If there is a defined allocation pattern, which is dictated by the trophic mode, this would help to understand the dominance of a trophic mode over another within the same species, under natural conditions. The dominance of one trophic mode may, in turn, profoundly affect trophic interactions between *Ochromonas* and its predators in nature.

Hypothesis 3. The biochemical composition of heterotrophic protists determines their nutritional quality for the rotifer *Keratella quadrata*.

With this hypothesis I examined the second aspect of this thesis: whether the biochemical composition of heterotrophic protists determines their nutritional quality for zooplankton predators. Here I carried out population growth experiments, whereby the rotifer *Keratella quadrata* was offered four heterotrophic protist species as prey (*Balanion planctonicum*, *Urotricha farcta*, *Cyclidium* sp., and *Chilomonas paramecium*). The cryptomonad *Cryptomonas phaseolus* was used as a control prey of good nutritional quality. Population growth rates and egg numbers of the rotifer were evaluated for 5 days periods, and were subsequently correlated to the fatty acid, amino acid, and sterol composition of the protists (presented in chapters 2 and 3).

The biochemical composition of the protists was significantly correlated to the cumulative egg numbers of *Keratella* but not to population growth rates. This suggests that biochemical requirements for egg production differ from those for population growth, in the case of *Keratella* fed heterotrophic protists. These findings corroborate the view that growth, reproduction, and survivorship have different metabolic demands (Sterner and Hessen, 1994).

Among the biochemical compounds, which were significantly correlated to the egg production of *Keratella*, polyunsaturated fatty acids were by far the most important, evidenced by the higher number of significant correlations (a total of eight, including γ-linolenic acid, arachidonic acid, EPA, DHA, and DPA), as compared to those found for sterols (ergosterol, desmosterol, and stigmastanol) and amino acids (leucine only). This result suggests that fatty acid limitation surpass sterol or amino acid limitation for *Keratella*, since polyunsaturated fatty acids were the biochemical class that exhibited the largest differences among protists species (chapter 2). Supplementation tests can help to elucidate the limiting effects of specific PUFAs for *Keratella* (see hypothesis 4).

As no population growth of *Keratella* was observed when feeding the rotifers with bacterivorous protists, there must be at least one additional factor constraining growth of the rotifers, when these were fed bacterivorous protists. This suggests that single biochemical factors become limiting only under non-limitation by another factor. This is along the lines of the Liebig's Law of the Minimum (Von Liebig, 1855) that states that an organism will become limited by whatever resource that is in lowest supply compared to the organism's needs. Negative

effects of bacterivorous protists on the survival of *Keratella* individuals could have led to the negative growth rates of *Keratella*'s populations.

Conclusion: The answer to the question "Does the biochemical composition of heterotrophic protists determine their nutritional value as prey for the rotifer *Keratella quadrata*?" is "Yes, but not necessarily". Although we found a significant effect of biochemical compounds, especially fatty acids, on the egg production of the rotifers, there were no relationships between the protists' biochemistry and the rotifers' growth rates. Additionally the rotifer was not able to grow on a diet of bacterivores. This means that additional factors may be primarily limiting *Keratella* fed bacterivorous protists, according to the Liebig's Law of the minimum. The results suggest that the biochemistry is only one factor in a pool of factors driving the nutrition of *Keratella* fed heterotrophic protists.

Future Perspectives: The significant correlations observed between prey biochemical compounds and cumulative egg numbers of *Keratella* clearly indicate the necessity for testing limitation by single biochemical compounds for *Keratella*'s growth and reproduction. This is possible through supplementation of a poor quality diet, such as *Chilomonas* or *Cyclidium* with the target biochemical compound (see hypothesis 4). There is a need for working with axenically cultured prey organisms, such as osmotrophs. It would minimize possible harmful effects due to high bacterial densities on the survival of the rotifers, which may have led to the negative growth rates of *Keratella*'s population grown on bacterivorous protists.

Hypothesis 4. *Chilomonas paramecium* can be successfully supplemented with essential fatty acids (EPA and DHA). *Chilomonas paramecium* supplemented with EPA and DHA will enhance growth and reproduction of *Keratella quadrata*.

Given the correlative evidence between *Keratella*'s egg numbers and several polyunsaturated fatty acids, the need to test limiting effects of single fatty acids on *Keratella*'s growth and reproduction became clear. An efficient method to test limiting effects of single biochemical compounds is to supplement a poor quality diet with the target compound. However, such a method has not been tested for heterotrophic protists before.

I chose the polyunsaturated fatty acids EPA and DHA, based on current information concerning limiting effects of both fatty acids for zooplankton species (Von Elert, 2000; Müller-Navarra et al., 2004). I chose *Chilomonas paramecium*

to be supplemented with EPA and DHA because *Chilomonas* showed the lowest EPA concentrations among all heterotrophic protists I have analysed (chapter 2). Secondly, *Chilomonas* is of similar size and shape as the control alga *Cryptomonas*. Rotifers may exhibit different ingestion rates for protists of different size and shape (Bogdan and Gilber, 1982; Rothhaupt, 1990); thus, I minimized artefacts due to differential ingestion by choosing a target organism and a control of similar size and shape.

The supplementation technique proposed by Von Elert (2002) proved to be adequate for supplementing *Chilomonas* with defined concentrations of the polyunsaturated fatty acids EPA and DHA. Hence, the method allowed the evaluation of direct limiting effects by specific fatty acids on the performance of *Keratella* fed *Chilomonas*, qualitatively and quantitatively.

Feeding on *Chilomonas* supplemented with DHA slightly improved rotifers' population growth rates, but strongly enhanced egg production as compared to non-supplemented *Chilomonas*. EPA enriched *Chilomonas* did not significantly improve population growth rates and egg production of the rotifer. A slightly enhanced effect on population growth was observed when feeding the rotifers with *Chilomonas* previously incubated with BSA, but without fatty acids. This suggests limiting effects of certain amino acids for *Keratella* fed *Chilomonas*.

DHA may be, more than EPA, the limiting factor in the interface bacterivorous protists – zooplankton. Dietary DHA has been found in high amounts in copepod's eggs (Sargent and Falk-Petersen, 1988). In some crustaceans DHA has been found in 2 – 5 fold higher amounts in eggs and ovaries than in other female tissues (Hayashi, 1976), indicating the importance of DHA for embryonic survival and growth. DHA is also believed to play an important role during larval development and metamorphosis of marine molluscs (Delaunay et al., 1993) and to promote enhanced larval growth of the zebra mussel *Dreissena polymorpha* (Wacker et al., 2002).

When testing different protists as prey for *Keratella* (chapter 5) egg production did significantly correlate with biochemical composition of the protists, whereas population growth did not. This trend was also observed here, when testing single fatty acids. The fact that DHA supplementation lead to increased egg numbers but only slightly enhanced *Keratella* population growth rates confirmed my previous results, that reproduction and population growth have different metabolic demands, in accordance with the stoichiometric theory (Sterner and Hessen, 1994).

The most important result here was that *Chilomonas* supplemented with fatty acid concentrations similar to those found in the algae *Cryptomonas* did not support population growth and egg production of *Keratella* as *Cryptomonas* did. Although supplementation has enhanced the nutritional quality of *Chilomonas* for *Keratella*, this heterotrophic flagellate was still of poor quality when compared to the algae. Hence, additional factors, such as other biochemical compounds, but perhaps also minerals as well as synergetic and antagonistic interactions among biomolecules may limit the performance of *Keratella* grown on a *Chilomonas* diet.

Conclusion: The question "Does the supplementation of *Chilomonas paramecium* with essential fatty acids enhance their nutritional value for *Keratella quadrata*?" can be answered with "YES, it does" but only with respect to DHA. DHA may primarily limit the nutritional quality at the interface bacterivorous protists – rotifers. Nevertheless, DHA supplementation of *Chilomonas* did not result in a nutritional quality comparable with that of the algae *Cryptomonas*.

Future Perspectives: An important approach should be to combine different biochemical compounds as well as mineral and biochemical supplementation to test for limitation of one compound under different concentrations of another. This would help to clarify, under which set of conditions a biochemical compound will limit the nutrition of *Keratella* fed heterotrophic protists.

Biochemical composition and nutritional quality of protists

To search for the biochemical or mineral compound, which primarily limits nutrition of a predator, is like looking for a needle in a haystack. Current methodological assays enable us to test the nutritional quality of different prey organisms and the results obtained often provide correlative evidence for certain compounds, which are likely candidates of causing limitation for a predator. As a next step, single compounds, for which correlative evidence was found, are directly tested through supplementation techniques. This methodological approach is very efficient for providing insights on the nutritional quality of a prey in a defined "prey – predator" model, and under a defined set of conditions. However, by using this method we will probably never exactly and undoubtfully identify that compound, which is primarily limiting a predator, because limitation may change with altering environmental conditions and among different prey-predator systems. This assumption is sustained by findings of contrasting limiting factors for a same predator species. For *Daphnia galeata* different biochemical and

mineral components have been claimed to be the "most important limiting factors" (Müller-Navarra, 1995; Weers and Gulati, 1997; Von Elert and Wollfrom, 2001; Von Elert, 2002). Such contrasting findings probably relate to different experimental conditions, as different prey organisms were used, cultures were submitted to different physical conditions, or the daphniids were isolated from different environments. Maybe we have been formulating the wrong question. Instead of searching for that one factor, which primary limits growth and reproduction of zooplankton organisms, efforts should be directed to understand under which set of conditions one single biochemical compound may be limiting. That some fatty acids or amino acids are essential for aquatic invertebrates is well known. A more realistic question could be: under which set of conditions a certain fatty acid or amino acid may play a more important limiting effect?

Müller-Navarra et al. (2004), for instance, elegantly showed that the degree of EPA limitation for *Daphnia* growth in nature depends on the phosphorus levels in the system. In phosphorus-poor lakes, sestonic EPA was able to efficiently limit growth rates of *Daphnia* populations. In phosphorus-rich systems, EPA limitation could not be discriminated from other limitation sources. Becker and Boersma (2003) showed that limitation effects of EPA on *Daphnia* life history could only be detected below a specific prey nutrient threshold. Above this threshold, the daphniids were limited by nutrient ratios. Both results make clear that limitation of a single factor depends on a constellation of factors, even for the same predator species.

I was first surprised to find no correlative evidence between the biochemical composition of the heterotrophic protists and population growth of *Keratella quadrata*. To my knowledge, the present study includes the widest range of biochemical compounds to test the nutritional quality of heterotrophic protists. Nevertheless, I did not find any evidence about the factors, which may have limited population growth of *Keratella*. It seems likely that the lower concentrations and relative amounts of several biochemical compounds (e.g. amino acids and sterols) found in the good quality algae *Cryptomonas* as compared to *Balanion* and *Urotricha* were already adequate for supporting *Keratella*'s population growth. If so, why did *Chilomonas* not support population growth of *Keratella*? *Chilomonas* had concentrations and relative amounts of several biochemical compounds similar to those found in *Cryptomonas*. And even when this was not the case (e.g. DHA), artificial supplementation did not enhance the nutritional quality of *Chilomonas* equivalent to that found for *Cryptomonas*. It is likely that a non-chemical harmful effect on *Keratella*'s survival, caused by high bacterial densities in the treatments (personal observation), has resulted in

the observed negative growth rates of the rotifers. Given that, I could not include bacterivores into the statistical analysis, which were performed for the algivores only. This reduced statistical variability may have contributed to the absence of significant relationships between *Keratella*'s growth rate and prey biochemistry. Working with axenic cultures (e.g. osmotrophs) would minimize such undesirable experimental artifacts.

Despite the absence of correlations between prey biochemical compounds and population growth rates of *Keratella*, the strong positive correlations found with egg production of *Keratella* suggests that the biochemistry of the protists, especially fatty acids, is indeed an important factor limiting *Keratella*'s performance.

The variation in terms of absolute concentrations found for polyunsaturated fatty acids in protists grown on different diets (algivores/bacterivores) and within a protist species grown under different trophic modes (autotrophic, mixotrophic, heterotrophic) suggests that polyunsaturated fatty acids most likely limit life history-traits of a predator fed protists. Fatty acid limitation for zooplankton has been observed for many species (Ahlgren et al., 1990; Brett and Müller-Navarra, 1997; Weers and Gulati, 1997; Becker and Boersma, 2003), mostly feeding on algae. I found positive relationships between several polyunsaturated fatty acids, such as γ-linolenic acid, EPA, DHA as well as arachidonic acid, and the egg production of *Keratella quadrata* (chapter 5). The number of polyunsaturated fatty acids which were significantly correlated with *Keratella*'s egg production was much higher than the number of sterols and amino acids. This probably means that polyunsaturated fatty acids are most likely limiting factors for *Keratella* feeding on heterotrophic protists. However, not only a single fatty acid is likely to be responsible for the entire limitation of *Keratella* fed heterotrophic protists, as suggested by the findings on *Chilomonas* supplemented with DHA (chapter 6). Probably a combination of biochemical compounds was responsible for the higher nutritional quality of algivores as compared to bacterivores. This could be tested by supplementing *Chilomonas* and *Cyclidium* with other single biochemical compounds, as well as with combined supplements of several fatty acids, sterols, and amino acids.

In this study, I showed that the biochemical composition of protists may be influenced by the dietary sources, but the biochemical concentrations are more dependent of metabolic features of the protists, such as efficient accumulation and synthesis. Moreover, the trophic mode exhibited by a protist species has an important effect on its lipid composition, especially that of fatty acids. It was

shown that *Keratella* was limited by the biochemical composition of heterotrophic protists. A direct test using supplementation of DHA confirmed the correlative evidence found for the limiting effects of DHA on *Keratella*'s reproduction, when the rotifers fed on bacterivorous protists.

My results on the biochemical composition of protists highlight the necessity to incorporate heterotrophic protists in food quality studies, because of their ability to modify the biochemical composition at an early stage in aquatic food webs, i.e. at the interface between algae/bacteria and the mesozooplankton. Considering the high energy density and essentiality of some biochemical compounds (such as lipids), small biochemical modifications at this early stage may have profound consequences for matter and energy transfer through the entire food web.

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9. TABLES

Table 1. Carbon-specific fatty acid concentrations (μg mgC^{-1}) in four freshwater heterotrophic protists and their diet. *Balanion planctonicum* and *Urotricha farcta* fed *Cryptomonas phaseolus; Cyclidium sp.* and *Chilomonas paramecium* fed a "bacteria + rice" diet. Values are means (±SD) of at least 4 analyses (n= 4); (-) not detected fatty acids.

Fatty Acid	*Cryptomonas* (algal diet)	*Balanion*	*Urotricha*	"Bacteria + rice" diet	*Cyclidium*	*Chilomonas*
14:0	7.8 (8.1)	2.6 (1.1)	2.6 (0.7)	-	0.6 (0.0)*	0.5 (0.0)*
15:0	11.9 (2.3)	10.1 (3.1)	13.9 (2.5)	0.9 (0.2)	3.2 (0.1)*	4.5 (0.5)*
16:0	111.6 (39.7)	82.5 (32.1)	103.8 (3.2)	17.0 (5.4)	47.0 (2.5)*	47.0 (13.2)*
17:0	1.3 (1.0)	3.2 (0.0)	3.8 (0.0)	0.3 (0.1)	1.4 (0.1)*	0.7 (0.0)*
18:0	44.8 (24.2)	32.8 (11.3)	40.7 (3.1)	7.2 (1.6)	24.4 (2.3)*	15.9 (1.4)*
19:0	-	23.5 (9.5)*	25.5 (12.8)*	4.8 (0.8)	19.4 (0.6)*	8.4 (0.2)*
20:0	8.3 (2.4)	6.2 (3.0)	4.7 (1.6)	0.9 (0.2)	3.7 (0.5)*	3.1 (0.7)*
24:0	12.2 (7.9)	7.2 (4.3)	2.9 (1.1)*	53.6 (5.7)	2.5 (2.2)*	0.7 (0.3)*
Summe SAFA	**207.3 (41.2)**	**202.4 (77.5)**	**235.1 (36.4)**	**35.4 (10.6)**	**116.7 (13.7)**	**98.9 (14.2)**
14:1 ω 7	1.9 (1.3)	11.7 (4.5)*	13.8 (2.9)*	3.0 (1.9)	4.2 (0.2)	5.8 (3.2)
14:1 ω 5	5.5 (1.1)	4.4 (1.2)	7.7 (0.8)*	1.9 (0.5)	5.9 (0.2)*	1.7 (0.5)
15:1	4.2 (1.4)	25.1 (9.6)*	27.1 (10.5)*	3.1 (0.9)	8.2 (0.3)*	0.5 (0.1)*
16:1 ω 9	7.0 (2.8)	20.3 (10.0)*	23.5 (1.5)*	6.3 (1.5)	17.2 (0.4)*	12.8 (1.3)*
16:1 ω 5	2.6 (2.5)	25.8 (8.2)*	29.1 (13.3)*	3.2 (0.9)	9.6 (0.1)*	9.3 (5.2)
17:1	1.4 (0.6)	13.0 (4.4)*	16.4 (9.4)*	2.5 (0.2)	10.3 (0.4)*	5.0 (0.7)*
18:1 ω 9	7.4 (5.8)	25.8 (10.9)*	40.4 (7.7)*	3.4 (0.8)	16.7 (1.5)*	25.3 (10.3)*
18:1 ω 7	1.5 (0.9)	14.8 (5.2)*	14.3 (5.8)*	1.0 (0.5)	33.5 (1.9)*	26.1 (5.0)*
20:1 ω 9	2.9 (1.5)	16.9 (2.4)*	17.0 (7.7)*	4.4 (2.1)	14.2 (2.7)*	-*
Summe MUFA	**56.7 (23.6)**	**176.9 (56.1)**	**212.9 (47.9)**	**31.1 (9.1)**	**125.8 (4.6)**	**93.3 (15.1)**
16:2 ω 4	13.9 (10.4)	90.1 (50.2)*	75.9 (53.8)*	17.5 (3.2)	36.3 (2.1)*	31.4 (2.8)*
16:3 ω 4	4.8 (3.8)	7.7 (2.3)	7.3 (1.5)	0.4 (0.0)	4.0 (0.2)*	1.7 (0.3)*
18:2 ω 6	6.5 (4.0)	25.9 (13.4)*	50.9 (12.5)*	3.3 (0.3)	8.0 (1.0)*	27.3 (17.6)*
18:3 ω 6	3.8 (1.3)	4.0 (0.0)	7.2 (3.2)	-	1.7 (0.2)*	1.2 (0.7)*
18:3 ω 4	2.0 (1.1)	2.1 (0.2)	3.8 (0.8)*	-	0.6 (0.1)*	3.9 (1.7)*
18:3 ω 3	1.1 (0.5)	7.5 (3.3)*	9.3 (2.9)*	1.6 (0.4)	2.7 (0.3)	3.4 (0.9)*
18:4 ω 3	2.2 (0.6)	14.3 (5.3)*	16.5 (6.9)*	2.2 (0.4)	8.5 (0.3)*	17.4 (14.0)*
20:2 ω 6	10.9 (7.6)	12.8 (4.6)	13.3 (11.9)	-	3.8 (0.3)*	18.9 (17.3)*
20:3 ω 6	10.0 (4.4)	-*	6.7 (6.1)	-	0.7 (0.1)*	0.9 (0.5)*
20:4 ω 6	8.9 (8.9)	2.2 (1.0)	4.2 (0.0)	0.7 (0.3)	1.5 (0.3)*	1.2 (0.2)
20:3 ω 3	1.5 (0.9)	3.3 (0.1)	5.3 (4.1)	1.1 (0.3)	2.0 (0.2)*	1.6 (0.2)
20:4 ω 3	13.2 (3.5)	7.6 (2.1)	5.3 (0.0)	1.3 (0.4)	2.8 (0.2)*	14.1 (12.2)*
20:5 ω 3 (EPA)	11.2 (5.8)	9.1 (1.9)	10.8 (5.1)	4.7 (0.7)	8.2 (0.2)*	4.6 (0.5)
21:5 ω 3	20.7 (2.0)	-	-	-	0.4 (0.0)*	0.6 (0.0)*
Summe 22 (ω 6)	1.6 (1.4)	16.7 (7.7)*	17.5 (4.0)*	2.2 (0.5)	13.3 (1.9)*	7.7 (0.7)*
22:6 ω 3 (DHA)	40.2 (6.3)	-*	-*	-	-	1.1 (0.69)*
Summe PUFA	**194.6 (106.4)**	**202.2 (92.9)**	**224.9 (86.5)**	**36.4 (5.8)**	**101.9 (11.3)**	**146.6 (72.6)**
ω6/ω3	0.7 (0.1)	1.3 (0.1)*	2.1 (0.3)*	0.5 (0.1)	0.9 (0.1)*	0.7 (0.4)

*Significant differences between protist fatty acid composition and the composition of its respective diet, according to one-way ANOVA followed by Dunnett's - test (P<0.05).

Table 2. Standardized canonical coefficients for each fatty acid in the discriminant functions (canonical roots) extracted by stepwise discriminant analysis (SDA), performed to identify fatty acids (independent variables), which significantly separate organisms (groups); Eigenvalues (Eigenv.), significance provided by Chi^2 - test (*P* value), and explained cumulative variance (Cum. r^2) are provided for each root following the fatty acids. Fatty acids marked with an asterisk discriminate organisms (groups) within a root. See Fig. 1 (chapter 2) for group's separation.

	Root				Root				Root		
SAFA	1	2	3	**MUFA**	1	2	3	**PUFA**	1	2	3
24:0	1.50*	0.17	0.29	**18:1ω7**	-3.02*	0.93	-0.01	**22:3ω6**	-8.09*	-1.178	-0.50
8:0	-1.13	0.47	1.95*	**12:1**	1.71	1.80*	0.30	**18:2ω6**	31.51*	6.73	3.52
19:0	1.60	-2.48*	-0.67	**14:1ω5**	-1.29	-1.42*	-0.93	**22:5ω3**	-3.39*	1.03	1.95
10:0	-0.48	-0.98	-1.01*	**16:1ω7**	1.96	2.00*	0.18	**22:5ω6**	-1.98	0.12	-0.96
20:0	1.32*	1.61	-0.09	**18:1ω9**	-3.09	-1.41	-0.18	**18:3ω6**	-19.2*	-7.70	-6.37
15:0	-0.40	0.39	0.86*	**22:1ω11**	3.63*	-0.29	0.37	**20:2ω6**	-38.7*	-2.17	-6.83
12:0	0.69	1.83*	-0.27	**16:1ω5**	2.60*	-0.63	1.23	**22:2ω6**	0.48	-5.65*	-0.41
21:0	0.89	-2.47*	-0.93	**20:1ω9**	0.23	0.46	-0.81*	**20:3ω3**	3.14	-2.23	3.25*
14:0	1.35*	1.08	0.49	**16:1ω9**	1.00	0.73	-0.13	**DPA**	8.06	15.49*	6.43
22:0	0.54*	-0.17	-0.17	**20:1ω11**	-0.85	1.54	0.12	**DHA**	20.38	-21.9*	-1.31
				24:1ω9	0.02	-0.25	0.24	**20:4ω3**	24.77*	-7.46	4.31
				15:1	0.32	-1.40	-0.65	**18:3ω3**	-7.16*	1.64	-1.58
								16:3ω4	-0.34	7.30*	4.34
								16:2ω4	-0.75	-6.07*	-2.40
								20:3ω6	-1.32	-3.36*	-1.52
								EPA	4.10	2.84	4.32*
								20:4ω6	-12.9	-1.05	-4.50
								18:3ω4	0.13	0.35	-1.94*
Eigenv.	128.2	26.4	17.5		241.7	34.2	5.8		5478.8	666.3	25.1
***P* value**	<0.01	<0.01	<0.01		<0.01	<0.01	<0.01		<0.01	<0.01	<0.01
Cum. r^2	0.72	0.87	0.97		0.84	0.96	0.98		0.89	0.99	0.99

Table 3. Carbon-specific amino acid concentrations (pMol fgC^{-1}) in four freshwater heterotrophic protists and their diet. *Balanion planctonicum* and *Urotricha farcta* fed *Cryptomonas phaseolus*; *Cyclidium sp.* and *Chilomonas paramecium* fed a "bacteria + rice" diet. Values are means (±SD) of at least 4 analyses (n= 4).

Amino acids	*Cryptomonas* algal diet	*Balanion*	*Urotricha*	"Bacteria + rice" diet	*Cyclidium*	*Chilomonas*
Histidine	0.03 ± 0.01	0.9 ± 0.3*	0.4 ± 0.3	0.01 ± 0.007	0.4 ± 0.2	1.9 ± 0.7*
Threonine	0.2 ± 0.2	6.3 ± 0.8*	4.0 ± 1.1*	0.09 ± 0.04	6.7 ± 4.4*	11.0 ± 3.9*
Arginine	0.2 ± 0.1	2.5 ± 0.5*	2.7 ± 1.5*	0.03 ± 0.01	3.0 ± 1.3*	6.9 ± 1.6*
Tryptophan	2.2 ± 1.3	5.0 ± 0.9	11.1± 3.0*	0.71 ± 0.53	15.5±11.4*	36.0 ± 6.6*
Methionine	0.1 ± 0.0	1.6 ± 0.9*	1.2 ± 0.2*	0.04 ± 0.004	2.0 ± 1.8	3.8 ± 1.5*
Valine	0.2 ± 0.1	1.3 ± 1.4	1.0 ± 0.7	0.01 ± 0.004	0.7 ± 0.1*	2.1 ± 0.2*
Phenylalanine	0.4 ± 0.2	2.0 ± 0.2*	3.0 ± 0.6*	0.05 ± 0.01	3.8 ± 2.7*	7.6 ± 1.8*
Isoleucine	0.2 ± 0.04	1.8 ± 0.9*	1.7 ± 0.5*	0.04 ± 0.01	2.5 ± 1.5*	6.2 ± 0.4*
Leucine	0.8 ± 0.1	18.5±2.5*	4.2 ± 0.6*	0.23 ± 0.12	7.4 ± 5.1*	3.6 ± 1.4*
Lysine	0.4 ± 0.3	0.7 ± 0.2	1.3 ± 0.5*	0.06 ± 0.02	1.8 ± 1.2*	4.5 ± 0.6

* Significant differences between protist amino acid composition and the composition of its respective diet, according to one-way ANOVA followed by Dunnett's - test (P<0.05).

Table 4. Standardized canonical coefficients for each amino acid in the discriminant functions (canonical roots) extracted by the stepwise discriminant analysis (SDA) performed to identify amino acids (independent variables), which significantly separate organisms (groups); Eigenvalues (Eigenv.), significance provided by Chi^2 - test (*P* value), and explained cumulative variance (Cum. r^2) for each discriminant function (canonical root) are provided for each root following the amino acids. Amino acids marked with an asterisk discriminate organisms (groups) within a root. See Fig. 2 (chapter 2) for group's separation.

Amino acid	**Root 1**	**Root 2**
Leucine	-0.747	-0.797*
Isoleucine	2.829*	0.273
Histidine	0.002	-1.220*
Phenylalanine	-8.559*	0.331
Tryptophan	5.086*	2.410
Lysine	5.168*	-3.178
Valine	-2.326*	-0.428
Methionine	-4.088*	-0.347
Arginine	-1.174	0.659
Eigenv.	**97.694**	**23.706**
***P* value**	**< 0.001**	**< 0.001**
Cum. r^2	**0.786**	**0.976**

Table 5. Sterol composition of the algal diet *Cryptomonas phaseolus* and the heterotrophic protists *Balanion planctonicum* and *Urotricha farcta*.

Sterol	***Cryptomonas phaseolus* (algal diet)**	***Balanion planctonicum***	***Urotricha farcta***
Cholesterol	6.5 ± 4.9	1.9 ± 0.6	5.1 ± 0.2
	(0.51±0.2)	(0.50±0.1)	(1.24±0.9)
Dihydrocholesterol	2.3 ± 2.1	-*	-*
	(0.36±0.3)		
Desmosterol	12.4 ± 4.6	18.4* ± 3.4	8.3 ± 0.7
	(7.13±1.4)	(6.44±1.0)	(2.39*±1.2)
Campesterol	1.5 ± 1.6	5.2* ± 1.8	0.3 ± 0.1
	(0.22±0.2)	(1.72*±0.7)	(0.11±0.0)
Stigmasterol	9.8 ± 2.0	49.3† ± 1.8	26.7† ± 8.2
	(0.85±0.5)	(16.81*±0.7)	(5.89*±3.6)
Sitosterol	0.6 ± 0.1	0.9 ± 0.4	6.1* ± 1.0
	(0.18±0.1)	(0.25±0.1)	(2.23†±0.6)
Lathosterol	0.5 ± 0.6	-*	0.1 ± 0.0
	(0.09±0.0)		(0.04±0.0)
Ergosterol	42.8 ± 21.8	-*	20.9 ± 5.9
	(10.18±0.8)		(2.98*±2.9)
Stigmastanol	5.13 ± 0.06	-*	0.9* ± 0.4
	(0.49±0.2)		(0.30±0.0)
Brassicasterol (%)	15.9 ± 4.8	20.6 ± 4.8	14.6 ± 1.9
24-Stigmasta-5,7,24(28)-trien-3β-ol (%)	-*	3.2 ± 1.7	13.9 ± 8.7
Undentified sterol (%)	-*	5.2 ± 0.6	3.1 ± 2.3
TOTAL (μg mgC^{-1})	**26.8±21.2**	**27.6±2.2**	**20.6±12.1**
Sterol precursors and other neutral lipids			
Squalene (μg mgC^{-1})	1.5 ± 1.2	0.5 ± 0.0	1.4 ± 0.7
Cycloartenol (%)	0.8 ± 0.1	-*	0.5 ± 0.0
Tetrahymanol (%)	-	-*	-
Hopan-22-ol (%)	0.1 ± 0.0	-	1.1* ± 0.5
Hop-22(29)-ene (%)	-	-	-

Values for sterols are given as relative (mean percentages of the total sterol pool ± S.D.) and absolute concentrations (μg mgC^{-1}± S.D., values in parenthesis; N = 4–5); Neutral lipids are provided as percentages of the total neutral lipid fraction; -: not detected.
*Significant differences at 95% confidence when comparing protists and diet (Mann Whitney *U*-test);
†Significant differences at 90% confidence when comparing protists and diet (Mann Whitney *U*-test);

Table 6. Sterol composition of *Chilomonas paramecium*, *Cyclidium sp*., and their diet – "bacteria + polished rice" (*Chilomonas* diet) and "bacteria + unpolished rice" (*Cyclidium* diet).

Sterol	**Bacteria + polished rice (*Chilomonas* diet)**	***Chilomonas paramecium***	**Bacteria + unpolished rice (*Cyclidium* diet)**	***Cyclidium sp.***
Cholesterol	65.8 ± 18.5	3.4* ± 1.4	17.3 ± 0.9	70.6* ± 5.2
	(9.85±3.2)	(1.77*±0.9)	(15.58±0.1)	(31.29±14.8)
Dihydrocholesterol	13.5 ± 5.5	-*	0.3 ± 0.0	3.1* ± 1.5
	(3.91±0.0)		(0.27±0.0)	(2.09*±1.5)
Desmosterol	5.5 ± 3.9	2.5 ± 0.1	0.5 ± 0.0	1.2* ± 0.7
	(0.72±0.3)	(1.45†±0.3)	(0.42±0.0)	(0.36±0.3)
Campesterol	-	12.1* ± 6.1	12.7 ± 1.0	2.6* ± 1.0
		(6.18*±4.7)	(11.53±1.6)	(0.98*±0.2)
Stigmasterol	6.2 ± 0.9	39.7* ± 12.6	12.2 ± 0.1	3.5* ± 0.6
	(1.29±0.1)	(17.43*±7.3)	(11.00±0.6)	(2.30*±0.7)
Sitosterol	-	32.1* ± 10.2	49.9 ± 0.7	10.7* ± 0.7
		(15.85*±10.4)	(45.12±2.2)	(4.10*±2.3)
Lathosterol	2.6 ± 0.8	2.2 ± 0.3	0.5 ± 0.2	2.8* ± 0.9
	(0.39±0.1)	(1.23*±0.1)	(0.50±0.2)	(1.67†±0.4)
Ergosterol	-	8.5* ± 6.5	0.3 ± 0.0	-*
		(2.08*±0.7)	(0.25±0.1)	
Stigmastanol	10.9 ± 7.6	1.4* ± 1.2	6.4 ± 0.3	3.0* ± 1.6
	(2.38±0.0)	(0.67±0.8)	(5.76±0.6)	(1.20*±0.7)
Brassicasterol	-	3.7* ± 0.7	-	-
24-Stigmasta-5,7,24(28)-trien-3β-ol (%)	-	0.4* ± 0.1	-	-
Unidentified sterol (%)	-	-	-	-
TOTAL (μg mgC^{-1})	**16.5 ± 8.3**	**46.1* ± 19.1**	**90.4* ± 5.6**	**37.7 ± 20.7**
terol precursors and other neutral lipids				
Squalene (μg mgC^{-1})	5.2 ± 1.4	8.4 ± 5.6	2.5 ± 0.2	2.6 ± 1.3
Cycloartenol (%)	-	4.8* ± 1.3	-	0.8* ± 0.2
Tetrahymanol (%)	-	-	-	11.5* ± 4.7
Hopan-22-ol (%)	-	-	-	30.2* ± 3.5
Hop-22(29)-ene (%)	-	-	-	2.5* ± 0.3

Values for sterols are given as relative (mean percentages of the total sterol pool ± S.D.) and absolute concentrations (μg mgC^{-1}± S.D., values in parenthesis; N = 4–5); Neutral lipids are provided as percentages of the total neutral lipid fraction; -: not detected.

*Significant differences at 95% confidence when comparing protist and its diet (Mann Whitney *U*-test);

†Significant differences at 90% confidence when comparing protist and its diet (Mann Whitney *U*-test);

Table 7. Carbon-specific fatty acid concentrations (ng mgC^{-1}) of *Ochromonas* sp. of different trophic modes. Values are means (±S.D.) of at least 4 replicates.

	Ochromonas sp.			
Fatty Acid	Autotroph	Mixotroph	Heterotroph	Bacterial diet
14:0	4.4 (2.1)	7.0 (2.9)	3.1 (1.6)	1.5 (0.2)
15:0	8.6 (5.1)	22.0 (4.1)	3.1 (2.2)	0.9 (0.1)
16:0	140.4 (40.9)	284.7 (201.3)	22.2 (5.3)	12.8 (1.2)
17:0	35.7 (15.1)	56.6 (19.3)	1.3 (0.3)	1.0 (0.5)
18:0	15.7 (7.2)	145.2 (84.7)	18.9 (6.4)	5.1 (0.5)
19:0	-	18.2 (7.8)	0.4 (0.1)	-
20:0	19.6 (10.5)	35.0 (20.5)	-	-
24:0	53.9 (2.4)	54.1 (36.7)	34.5 (14.7)	13.4 (2.3)
Sum SAFA	**379.6 (139.5)**	**893.8 (59.5)**	**108.2 (41.3)**	**37.1 (1.2)**
14:1 ω 7	4.4 (1.7)	1.9 (1.0)	1.4 (0.5)	0.4 (0.1)
14:1 ω 5	6.6 (2.6)	5.2 (2.6)	6.5 (1.7)	2.9 (0.3)
15:1	5.4 (3.2)	142.2 (59.2)	2.4 (0.8)	1.0 (0.1)
16:1 ω 9	8.6 (6.3)	-	0.5 (0.3)	0.3 (0.1)
16:1 ω 5	8.1 (3.1)	5.4 (3.0)	0.5 (0.2)	0.8 (0.2)
17:1	78.8 (17.7)	63.7 (24.6)	13.9 (3.9)	8.2 (1.5)
18:1 ω 9	14.7 (7.9)	7.9 (3.2)	10.4 (7.4)	37.2 (9.9)
18:1 ω 7	20.7 (9.1)	2.9 (2.3)	6.8 (2.0)	3.9 (0.7)
20:1 ω 9	14.5 (9.8)	-	-	-
20:1 ω 11	7.2 (4.1)	9.9 (1.8)	-	-
22:1 ω 9	66.0 (24.0)	65.8 (24.8)	-	-
Sum MUFA	**405.8 (199.7)**	**460.8 (156.7)**	**68.9 (44.1)**	**57.4 (12.0)**
16:2 ω 4	21.7 (10.0)	21.1 (5.0)	1.2 (0.5)	3.2 (1.4)
16:3 ω 4	15.5 (4.1)	9.2 (4.7)	3.1 (1.2)	2.5 (0.0)
18:2 ω 6	18.9 (5.9)	12.4 (10.2)	7.3 (3.4)	3.9 (0.7)
18:3 ω6	19.1 (4.3)	5.5 (2.5)	2.0 (0.8)	1.4 (0.5)
18:3 ω 4	-	7.4 (2.0)	-	-
18:3 ω 3	12.0 (6.4)	5.4 (1.6)	2.0 (1.2)	1.5 (0.9)
18:4 ω 3	39.6 (15.2)	2.0 (1.2)	9.0 (2.2)	4.5 (0.9)
20:2 ω6	26.0 (18.0)	51.6 (18.6)	-	-
20:3 ω6	51.9 (35.2)	55.7 (26.8)	-	-
20:4 ω 6	17.0 (13.9)	-	-	-
20:3 ω 3	4.1 (1.3)	16.9 (10.6)	-	-
20:4 ω 3	44.0 (19.5)	-	-	-
20:5 ω 3 (EPA)	16.2 (8.5)	67.7 (12.0)	-	-
21:5 ω 3	113.4 (47.8)	40.8 (24.4)	-	-
22:5 ω 3	24.9 (1.7)	-	-	-
22:6 ω 3	60.0 (3.2)	73.5 (1.4)	-	-
Sum PUFA	**620.5 (281.4)**	**369.2 (50.5)**	**33.2 (10.7)**	**20.0 (3.4)**
ω 6 / ω 3	0.4 (0.1)	0.8 (0.2)	0.7 (0.1)	0.9 (0.2)

The total sum of SAFA, MUFA, and PUFA takes other measured fatty acids into account, which were not presented here; -: not found.

Table 8. Standardized canonical coefficients for each fatty acid in the discriminant functions (canonical roots) extracted by discriminant analyses (DA) performed to identify fatty acids (independent variables), which significantly separate trophic modes (groups); Eigenvalues (Eigenv.), significance provided by Chi^2 - test (*P* value), and explained cumulative variance (Cum. r^2) are provided for each root. Fatty acids marked with an asterisk significantly discriminated trophic modes (groups) within a root. See Fig. 2 (chapter 4) for group's separation.

	Root			Root			Root	
SAFA	1	2	***MUFA***	1	2	**PUFA**	1	2
14:0	-0.08	0.09	**14:1ω7**	-0.73*	0.78	**16:2ω4**	-0.45	-2.14*
15:0	0.20	-0.18	**14:1ω5**	0.06	0.01	**20:3ω3**	-10.32*	-4.46
16:0	3.64*	1.68	**15:1**	1.80*	1.45	**DHA**	3.12	-3.76*
17:0	-0.38	-0.46	**16:1ω9**	-0.61	0.89	**18:3ω6**	3.42	-5.95*
18:0	-2.63*	-2.02	**16:1ω7**	-0.34	1.27	**18:4ω3**	0.37	3.14*
19:0	0.43	0.64	**16:1ω5**	-0.23	0.55	**20:3ω6**	-2.91	7.13*
20:0	-5.86*	0.16	**17:1**	-1.23	-2.10*	**22:5ω3**	-1.54	0.15*
21:0	6.67*	-0.51	**18:1ω9**	-0.17	-0.03	**16:3ω4**	14.01*	3.75
22:0	0.19	-0.17	**18:1ω7**	0.27	-0.33	**18:3ω3**	-18.76*	-11.66
24:0	-1.30*	0.63	**20:1ω9**	4.80*	-2.25	**18:3ω4**	5.97*	5.14
			20:1ω11	-3.23	5.12*	**EPA**	12.06*	8.55
			22:1ω9	-1.32	-2.53	**DPA**	0.01	2.98*
			24:1ω9	0.22	0.65	**20:4ω3**	-2.18	3.46*
Eigenv.	**13.93**	**1.92**		**8.21**	**1.11**		**377.71**	**29.12**
***P* value**	**<0.01**	**0.06**		**<0.01**	**<0.05**		**<0.01**	**<0.01**
Cum. r^2	**0.879**	**1.000**		**0.881**	**1.000**		**0.928**	**1.000**

Table 9. Carbon-specific sterol concentrations (ng mgC^{-1}) of *Ochromonas* sp. of different trophic modes. Values are means (±S.D.) from at least 4 replicates.

	***Ochromonas* sp.**			
Sterol	Autotroph	Mixotroph	Heterotroph	Bacterial diet
Cholesterol	3.8 (2.5)	0.1 (0.01)*	1.6 (0.2)	0.4 (0.2)
Dihydrocholesterol	-	-	-	-
Desmosterol	-	-	-	-
Campesterol	-	0.03 (0.01)	0.4 (0.2)*	0.04 (0.04)
Stigmasterol	38.9 (18.7)	22.8 (7.4)*	39.9 (9.2)	0.1 (0.1)
Sitosterol	2.1 (1.5)	0.2 (0.03)*	0.9 (0.3)	1.1 (1.0)
Lathosterol	0.2 (0.1)	0.02 (0.01)*	0.2 (0.1)	0.02 (0.01)
Ergosterol	-	-	-	-
Stigmastanol	-	-	-	-
Brassicasterol	-	-	-	-
24-Stigmasta-5,7,24(28)-trien-3β-ol[†] (%)	-	-	-	-
Unidentified sterol[†] (%)	1.5 (0.1)*	-	-	-
TOTAL (ng mgC^{-1})	**62.4 (41.0)**	**22.9 (7.4)**	**48.7 (17.3)**	**1.6 (1.2)**
Squalene (sterol precursor)	6.1 (1.1)*	1.6 (1.1)	2.2 (0.4)	0.7 (0.2)

Values for sterols are given as absolute concentrations (ng mgC^{-1} ± S.D; N = 4–8);
-: not detected.
[†]Quantitative analysis as percentages of the total neutral lipid fraction.
*Significant differences at 95% confidence (ANOVA and Tukey – *HSD* test);

Table 10. Standardized canonical coefficients for each sterol in the discriminant functions (canonical roots) extracted by the discriminant analysis (DA) performed to identify sterols (independent variables), which significantly separate trophic modes (groups); Eigenvalues (Eigenv.), significance provided by Chi^2 - test (*P* value), and explained cumulative variance (Cum. r^2) for each discriminant function (canonical root) are provided for each root. Sterols marked with an asterisk significantly discriminate trophic modes (groups) within each root. See Fig. 4 (chapter 4) for groups' separation.

Sterol	**Root 1**	**Root 2**
Cholesterol	1.53*	1.18
Lathosterol	-0.98	-1.18*
Stigmasterol	0.64	-0.54
Campesterol	-0.26	-0.60*
Sitosterol	0.93*	-0.01
Eigenv.	**2.284**	**1.022**
***P* value**	**< 0.001**	**0.018**
Cum. r^2	**0.690**	**1.000**

Table 11. Non-linear regression parameters calculated for the cumulative egg production (y) versus experimental time (x) (see chapter 5, Fig. 2 for fitting curves). Same letters indicate similar cumulative egg numbers when protist prey treatments are compared to the treatments with *C. phaseolus* and the control without prey (Dunnett's *t*-test).

Prey organism	Equation	Dunnett's *t*-test
Cryptomonas phaseolus	$y = - 0.28 + 6.33x \ln(x)$	A
Balanion planctonicum	$\ln(y) = 3.96 - 11.59e^{-x}$	A
Urotricha farcta	$y = - 16.10 + 15.79x^{0.583}$	B
Cyclidium sp.	$y = - 14.30 + 14.03x^{0.533}$	B
Chilomonas paramecium	$y = - 4.11 + 4.11x^{0.923}$	B
Control without prey	$y = - 2.46 + 2.35x^{0.472}$	C

Table 12. Spearman rank correlation coefficients (R) and significances (*P*), for cumulative egg numbers of *Keratella quadrata* versus prey fatty acid absolute concentration (conc.) and relative amounts (%). No significant relationship could be detected between *Keratella* population growth rates and prey biochemical composition.

Fatty acid	$R_{Spearman}$	*P*
Σ PUFA	0.45	0.01*
18:2ω6 (conc.)	-0.49	0.00*
18:3ω6 (conc.)	0.56	0.00*
18:4ω3 (conc.)	-0.58	0.00*
20:3ω6 (conc.)	0.54	0.00*
20:4ω6 (conc.)	0.69	0.00*
20:5ω3 (EPA) (conc.)	0.69	0.00*
21:5ω3 (conc.)	0.52	0.01*
% PUFA	0.78	0.00*
% 18:2ω6	-0.49	0.00*
% 18:3ω6	0.39	0.02*
% 20:3ω6	0.71	0.00*
% 20:4ω6	0.50	0.00*
% 22:2ω6	0.84	0.00*
% 22:3ω6	-0.56	0.01*
% 21:5ω3	0.52	0.01*
% 22:5ω6	0.65	0.00*
% 22:6ω3 (DHA)	0.84	0.00*

*Significant *P* values at 95% confidence

Table 13. Spearman rank correlation coefficients (R) and significances (*P*) for population growth rates and cumulative egg numbers of *Keratella quadrata* versus prey sterol and amino acid absolute concentration (conc.) and relative amounts (%). No significant relationship could be detected between *Keratella* population growth rates and prey biochemical composition.

Biochemical parameter	$R_{Spearman}$	*P*
Desmosterol (conc.)	0.67	0.00*
%Desmosterol	0.67	0.00*
Ergosterol (conc.)	0.79	0.00*
%Ergosterol	0.79	0.00*
%Stigmastanol	0.55	0.00*
%Leucine	0.77	0.00*

* Significant P values at 95% confidence

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As many people first read the acknowledgments section of a thesis (including me), I decided to make this section something very special. I should advice, that this is a brazilian acknowledgements section, which means: filled with emotion and … very, very long (my PhD colleagues would say “kitschig” or “peinlich”). I hope that all of you, which were mentioned here, will realize that your support was really very important to me.

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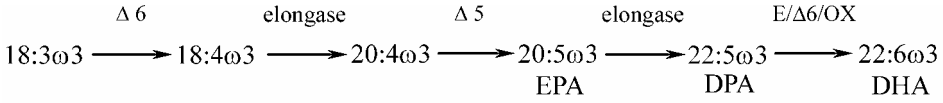
1.1 SYNTHESIS OF EPA AND DHA



The linolenic acid (18:3ω3) is converted to EPA through a series of elongation and desaturation reactions catalysed by different enzymes in animals and plants. In bacteria one enzyme is responsible for more reactions. DHA is converted from DPA through elongation, desaturation, and oxidations steps.

Enzymes:

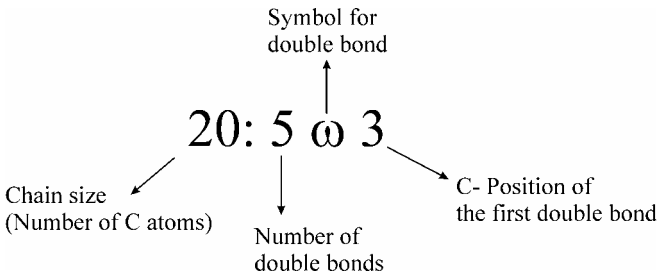
Δ6: delta-6 desaturase

Δ5: delta-5 desaturase

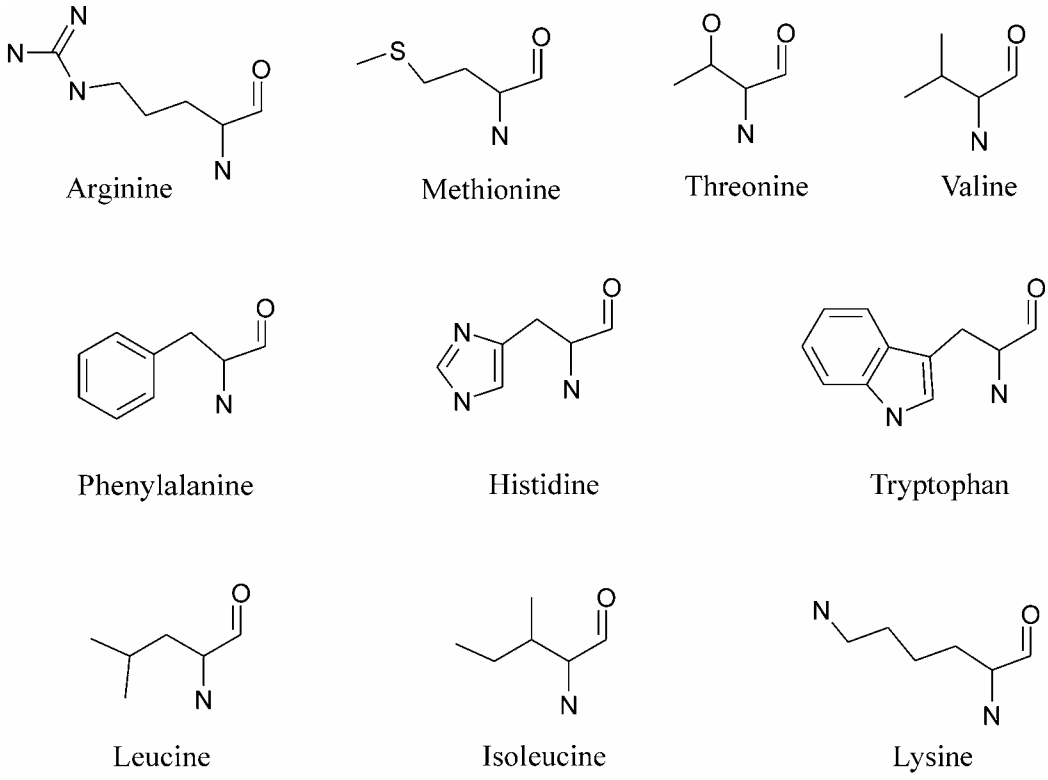
E: elongase

OX: oxidase

1.2 NOMENCLATURE OF FATTY ACIDS



APPENDIX 2. MOLECULAR STRUCTURE OF SOME ESSENTIAL AMINO ACIDS



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