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A biochemical explanation for the success of mixotrophy in the flagellate *Ochromonas* sp.

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Abstract

We report the influence of different nutritional modes—autotrophy, mixotrophy, and heterotrophy—on the fatty acid and sterol composition of the freshwater flagellate *Ochromonas* sp. and discuss the ecological significance of our results with respect to the resource competition theory (rct). Polyunsaturated fatty acids (PUFAs) are the most efficient biochemical variable distinguishing between nutritional modes of *Ochromonas* sp. Decreasing concentrations of PUFAs were observed in the order autotrophs, mixotrophs, heterotrophs. In mixotrophs and heterotrophs, concentrations of saturated fatty acids were higher than those of monounsaturated fatty acids and PUFAs as a result of bacterivory. Stigmasterol was the main sterol in *Ochromonas* sp., regardless of nutritional mode. Mixotrophs showed higher growth rates than heterotrophs, which could not be explained by rct. Heterotrophs, in turn, exhibited higher growth rates than autotrophs, which were cultured under the same light conditions as mixotrophs. Mixotrophs can synthesize PUFAs, which are important for many physiological functions such as membrane permeability and growth. Thus, mixotrophy facilitated efficient growth as well as the ability to synthesize complex and essential biomolecules. These strong synergetic effects are due to the combination of biochemical benefits of heterotrophic and autotrophic metabolic pathways and cannot be predicted by rct.

Living organisms require energy to drive the chemical reactions necessary for maintenance, growth, and reproduction. Energy is usually obtained by autotrophy or heterotrophy (Sanders 1991). Photoautotrophic primary producers use light energy to synthesize energy-rich organic molecules during photosynthesis. In contrast, heterotrophic organisms rely on the organic matter composition obtained from ingested particles (i.e., phagotrophy) or dissolved matter (i.e., osmotrophy). However, some organisms belong to more than one trophic level, since they combine the ability of photosynthesis and organic matter uptake, thus acting as both primary producers and consumers. This special nutritional strategy called mixotrophy has been considered a successful evolutionary strategy to overcome stress situations (Jones 1994; Raven 1997; Stoecker 1998). In aquatic flagellates, all of the nutritional modes seen among eukaryotes occur. Moreover, aquatic flagellates exhibit the largest number of species living mixotrophically, especially within the *Chrysophyta* (Sanders 1991). The chrysophyte flagellated genus *Ochromonas* is commonly found in a broad range of habitats in both marine and freshwater systems. It is able to cope with extreme environments, such as acidic water bodies (pH < 3.0; Wollmann et al. 2000), and has been successfully cultured under autotrophic, mixotrophic, and heterotrophic conditions (Rothhaupt 1996*a*,*b*; Sanders et al. 2001).

While autotrophic flagellates are able to synthesize all biochemical compounds necessary for their metabolic activities, heterotrophic flagellates are unable to synthesize most biochemical molecules (Sleigh 2000). Thus, they depend on the uptake of many classes of organic molecules from their environment. Indeed, first studies on the biochemical composition of heterotrophic protists have shown a certain dependency on dietary composition (Ederington et al. 1995); although more recent studies have revealed that species-specific metabolic features may lead to different fatty acid, amino acid, and sterol compositions, even among protist species fed the same diet (Boëchat and Adrian 2005; Boëchat et al. 2007).

Mixotrophs invest in both a photosynthetic apparatus and mechanisms of prey uptake and digestion. This may

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imply high energetic costs, but it can also represent an ecological advantage when light or nutrients are limited (Rothhaupt 1996*a*,*b*). However, the "shift" from one growth pathway to another has profound metabolic implications that drive adaptation (Sleigh 2000) and may result in altered biochemical composition, as reported for the fatty acid profile of the green alga *Chlamydomonas* sp. growing in acidic lakes (Poerschmann et al. 2004). Since the nutritional quality of planktonic prey organisms has been associated with the presence of essential biochemical compounds, such as highly unsaturated fatty acids and sterols (Brett and Müller-Navarra 1997; Hasset 2004), the nutritional mode of a protist may be expected to affect its nutritional quality as prey via changes in its biochemical composition.

Since the theory of the microbial loop was proposed by Azam et al. (1983), the significance of organic matter transfer between the microbial and the classic food web has become well recognized. However, understanding the ecological significance of biochemical transfer for carbon and energy fluxes between the microbial and the classical food web remains a challenge in aquatic ecology. Moreover, knowledge of the factors controlling biochemical composition and metabolism of aquatic protists becomes fundamental for understanding the mechanisms of protist competition (Rothhaupt 1996*a*) and distribution in situ. Therefore, we investigated how the nutritional mode of the freshwater flagellate *Ochromonas* sp. determines its biochemical profile and population growth. Qualitative and quantitative differences in the fatty acid and sterol composition among *Ochromonas* sp. cultured autotrophically, mixotrophically, and heterotrophically point to different metabolic abilities of this flagellate linked to its nutritional mode. Our results provide support for strong synergetic effects due to the combination of autotrophy and heterotrophy under limiting resource conditions that cannot be predicted by classical resource competition theory.

Materials and methods

Cultures—The flagellate *Ochromonas* sp. (length: 5–8 μm) was isolated from a mining lake (L111) in the region of Lusatia, Germany (Tittel et al. 2003). The lake is characterized by a pH of around 2.7 and high concentrations of iron and sulphate (Wollmann et al. 2000). *Ochromonas* sp. is the dominant flagellate, and its population maximum occurs in the epilimnion in summer. The prevailing nutritional mode of the mixotrophic flagellate *Ochromonas* sp. in the field is heterotrophy, potentially supported by autotrophy (Tittel et al. 2003). In the epilimnion, the heterotrophic carbon gain of *Ochromonas* sp. is three times higher than autotrophic production (Schmidtke et al. 2006). According to light-microscopic analysis the strain was identified as a member of the genus *Ochromonas* (Nixdorf et al. 1998); however, it was not possible to assign a species name. Phylogenetic analyses of the *rbcL* gene place our strain in a clade with *Tessellaria volvocina* (Playfair) Playfair, which is distantly related to typical *Ochromonas* species (e.g., *Ochromonas danica* Prings.) (R.A. Andersen, pers. comm.). Based on 18S rRNA analysis, it is adjacent to an environmental sequence from the acidic Tinto River in Spain (R.A. Andersen and M. Höfle, separate analyses, pers. comm.). The phylogeny of *Ochromonas* is currently under substantial revision (Andersen in press), and it has been suggested that the genus *Ochromonas* is polyphyletic (Andersen et al. 1999; Boenigk et al. 2005). We keep the name *Ochromonas* sp. for our strain until the taxonomy of the genus *Ochromonas* is revised. It is, however, the same strain used in previous studies (Tittel et al. 2003; Schmidtke et al. 2006).

Ochromonas sp. was cultured at 20°C in a medium free of organic carbon and with an ion composition and pH value (=2.65) reflecting that of the L111 (Bissinger et al. 2000). Three different kinds of cultures were set up in duplicate. For autotrophic growth, the cultures were kept in the light with photosynthetic available radiation of 70 μmol photons m^{-2} s^{-1} on a light : dark regime of 16 : 8 h. Seventy μmol photons m^{-2} s^{-1} is the photon flux density in the epilimnion on a bright summer's day at the bottom of the epilimnion (at a depth of 4.3 m) of L111, assuming an attenuation coefficient of 0.77 m^{-1} (Kamjunke et al. 2004). Although autotrophic cultures were not axenic, bacterial growth and abundance ($<10^5$ mL^{-1}) were very low, since the medium used was free of organic carbon. For mixotrophic growth, the same light conditions were applied, but bacteria isolated from L111 were added. The bacterial density used here (about 5×10^6 cells mL^{-1}) is higher than the average bacterial density observed in L111 ($3.38 \pm 1.09 \times 10^5$ mL^{-1}, Kamjunke et al. 2004) but does not promote maximal growth of *Ochromonas* sp. (Weithoff unpubl. data). Cultures of heterotrophs received the same bacterial density as those of mixotrophs but were kept in the dark. Bacteria were cultured in the same medium as *Ochromonas* sp., except for the addition of glucose to promote growth. The flagellates were mixotrophically cultivated for 2 weeks, autotrophically for 3 weeks, and heterotrophically for 6 weeks before initiating the experiments.

Sample preparation—Flagellate and bacteria cultures were filtered in duplicate on precombusted GF/C and GF/F-glass fiber filters, respectively (Whatman). The density of flagellates and bacteria was determined in both the stock 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 fluorescence microscope (Zeiss Axioscope 2). The carbon content of the bacteria was calculated from size measurements according to Simon and Azam (1989). *Ochromonas* sp. was measured by computer-aided image analysis (TSO). Cell volume was calculated assuming a rotational ellipsoid and converted into carbon units assuming a conversion factor of 0.23 pg C μm^{-3} (Kamjunke et al. 2004).

Fatty acid and sterol analyses—Filters were extracted in a 2 : 1 (v : v) chloroform : methanol solution (Folch et al. 1957) and homogenized for 5 min at 5,000 cycles min^{-1} (Ultraschall-Desintegrator USD 20, VDE Wiss. Gerätebau). Tricosanoic acid and 5α-cholestane (0.2 mg mL^{-1}) were used as internal standards for fatty acids and sterols,

respectively. Fatty acid methyl-esters (FAME) were formed by adding sulfuric acid (5% v : v) and heating the samples for 4 h at 80°C. Aliquots of 0.2 μL of the samples were injected into a Varian Star 3600 CX series gas chromatograph, equipped with a fused silica capillary column (Omegawax 320, SUPELCO, 30 m × 0.32 mm). Injector and flame ionization detector (FID) temperatures were 250°C and 260°C. Nitrogen was used as carrier gas. FAMEs were identified by comparing retention times with a standard solution (Supelco FAME Mix 47885-4, PUFA No. 3-47085-4, and PUFA No. 1-47033) and quantified by comparing peak areas with the internal standard area.

For sterol analysis, extracts were subjected to alkaline hydrolysis by the addition of 5 mL 1 mol L^{-1} potassium hydroxide (KOH) solution in 80% methanol (MeOH) and heating the samples at 80°C for 30 min (Gordon and Collins 1982). Free neutral lipids were silylated by adding 25 μL Bis (trimethylsilyl) trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane and 75 μL pyridine and heating the samples at 60°C for 30 min (Klein-Breteler et al. 1999). Sterol silylether derivatives were analyzed using a gas chromatograph Agilent 6890 equipped with a mass selective detector Agilent 5973-N (Waldbronn, Germany) and a fused silica capillary column HP-5MS (60 m × 0.32 mm × 0.25 μm). Helium was the carrier gas. The temperatures of the programmed temperature vaporization (PTV) inlet and of the detector interface were 300°C and 280°C, respectively (Boëchat et al. 2007). Sterol silylether derivatives were identified by their retention times and mass spectra in full scan mode previously calibrated with sterol standards (Sigma-Aldrich). Identified mass spectra were compared with mass spectra of a self-generated spectra library (Agilent Chemstation). Quantification was performed 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 sterols per milliliter injected sample.

Grazing and growth rates—Grazing rates of mixotrophs and heterotrophs on bacterial cells were estimated using the dilution method by Landry and Hasset (1982) modified by Landry (1993). From the stock cultures, four dilutions (75%, 50%, 25%, and 10%) were prepared in four replicates each. From each dilution and replicate as well as stock cultures, 100 mL were transferred into an Erlenmeyer flask with 20 mg C L^{-1} glucose to promote unlimited growth of bacteria and incubated for 24 h. The experiments were stopped by adding Lugol's iodine and sulphuric acid; the latter was used to avoid iron precipitation. The observed growth rates of bacteria (r) were calculated as

$$r = \frac{\ln P_{t1}/P_{t0}}{t_1 - t_0} \quad (1)$$

where P is the bacterial density at the beginning (t_0) and end (t_1) of the experiment. The growth rate r in each treatment is the difference of the instantaneous growth rate μ and the mortality (m) caused by flagellate grazing ($r = \mu - m$). The grazing mortality is calculated as

$$m = F[D] \quad (2)$$

where F is the volume swept clear of prey per grazer and time and $[D]$ is the time-averaged density of the grazer:

$$[D] = \frac{D_{t1} - D_{t0}}{\ln D_{t1}/D_{t0}} \quad (3)$$

where D is the grazer density. When μ and F are independent from the dilution, r varies linearly as a function of grazer density, with intercept μ and slope $-F$ (Landry 1993). Grazing rates (G) were calculated as

$$G = PF \quad (4)$$

Statistical analyses—Differences in fatty acid and sterol amounts among nutritional modes of *Ochromonas* sp. were tested with one-way analysis of variance (ANOVA) followed by the post hoc Tukey honestly significant difference (HSD) test. Percentage data were arcsine square-root transformed prior to ANOVA. To single out fatty acids and sterols that mostly differed among nutritional modes, discriminant analyses (DAs) were run separately for saturated fatty acids (SAFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), and sterols. Grazing and growth rates were compared using a Mann–Whitney U-test. Cell volumes were compared using one-way ANOVA, followed by the Tukey–HSD test. Normality of data distribution was assured by log-transforming cell volume data prior to analysis. All procedures were run in Statistica for Windows (version 5.01, Stat Soft).

Results

Fatty acids—Fatty acid relative composition differed among nutritional modes of *Ochromonas* sp. (Fig. 1). Autotrophic *Ochromonas* sp. exhibited higher percentages of PUFAs (44%) than mixotrophs (15%) and heterotrophs (16%) (ANOVA, $p < 0.01$, $n = 8$). Percentages of MUFAs were similar in autotrophs (21%), mixotrophs (28%), and heterotrophs (31%) (ANOVA, $p = 0.13$, $n = 8$). Lower percentages of SAFAs were found in autotrophs (28%) when compared with mixotrophs (64%) and heterotrophs (53%) (ANOVA, $p < 0.01$, $n = 8$). Mixotrophs and heterotrophs showed both higher SAFA and lower MUFA percentages than their bacterial diet (ANOVA, $p < 0.01$, $n = 8$). Total PUFA percentages in mixotrophs and heterotrophs reflected the percentages found in the bacteria (ANOVA, $p = 0.84$, $n = 8$).

Differences in absolute concentrations of individual fatty acids also reflected the nutritional mode of *Ochromonas* sp. (Table 1). Autotrophs and mixotrophs had higher concentrations of the SAFAs 16:0, 17:0, and 18:0 than heterotrophs (Table 1). The SAFA 20:0 was not detected in heterotrophs, but small amounts of the SAFA 19:0, which was not observed in the bacterial diet, were present in heterotrophs. The concentrations of the MUFAs 15:1, 16:1ω5, and 17:1 were higher in autotrophs and mixotrophs than in heterotrophs (Table 1). Heterotrophs were rich in the MUFA 18:1ω9, which was also found in high concentrations in the bacterial diet. The MUFA 22:1ω9 was only found in autotrophs and mixotrophs. The highest

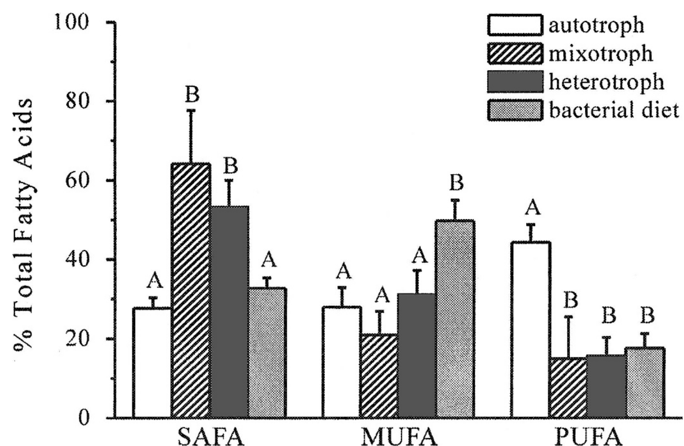

Fig. 1. Percentages (±SD) of saturated (SAFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids in the total fatty acid content of autotrophic, mixotrophic, and heterotrophic *Ochromonas* sp., as well as in the bacterial diet of heterotrophic and mixotrophic *Ochromonas* sp. Different letters indicate significant differences between variables at $p < 0.05$ (ANOVA and Tukey–HSD test).

differences in individual fatty acids were observed for PUFAs. Heterotrophs did not have PUFAs with carbon chains longer than 18 atoms. Moreover, absolute concentrations of all PUFAs present in heterotrophs reflected the concentrations found in the bacterial diet (Table 1). Autotrophs and mixotrophs in turn contained high concentrations of long-chain PUFAs (with more than 20 carbon atoms chain length), especially of 20:3ω6, 21:5ω3, and 20:5ω3 eicosapentaenoic acid (EPA) for mixotrophs and 22:6ω3 docosahexaenoic acid (DHA) for autotrophs (Table 1). Ratios of ω6 to ω3 fatty acids were similar in mixotrophs and heterotrophs, and higher than in autotrophs (ANOVA, $p < 0.01$, $n = 8$). Overall, heterotrophically growing flagellates had much lower total fatty acid contents than autotrophically and mixotrophically growing flagellates (Table 1).

The DAs run separately for SAFAs, MUFAs, and PUFAs provided fatty acid combinations that distinguished most efficiently among the nutritional modes of *Ochromonas* sp. (Fig. 2). For SAFAs, only the first root was significant (Wilk's Lambda = 0.023, $F_{26,16} = 6.16$, $p < 0.001$). All nutritional modes were separated at this root, but the best separation was observed between autotrophs and heterotrophs, whereas mixotrophs occupied an intermediate position. Differences in the SAFAs 16:0, 18:0, 20:0, 21:0, and 24:0 drove the separation at this root. The DA on MUFAs provided two significant roots (Wilk's Lambda = 0.008, $F_{26,16} = 6.01$, $p < 0.001$). The first root separated mixotrophs from autotrophs and heterotrophs because of differences in 15:1 and 20:1ω9 concentrations. The second root separated autotrophs from heterotrophs because of differences in 17:1, 20:1ω11, and 22:1ω9 concentrations. PUFAs were the most efficient molecules in separating *Ochromonas* sp. according to the nutritional mode (Fig. 2), as revealed by two significant roots extracted by the DA (Wilk's Lambda = 0.0004, $F_{26,16} = 31.09$, $p < 0.001$). The first root separated mixotrophs because of differences in 20:3ω3, 16:3ω4, 18:3ω3, 18:3ω4, and EPA concentrations. The second root separated heterotrophs from autotrophs because of the PUFAs 16:2ω4, DHA, 18:3ω6, 18:4ω3, 20:3ω6, 22:5ω3, 21:5ω3, and 20:4ω3.

Sterols—Total sterol concentration did not differ significantly among *Ochromonas* sp. of different nutritional mode, although significant differences were found for single sterols (Table 2). Overall, mixotrophs had the lowest absolute sterol concentrations among the flagellates (ANOVA, all $p < 0.05$, $n = 8$). The only exception was stigmasterol, which was similar in mixotrophs and autotrophs (ANOVA, $p < 0.05$, $n = 8$). Concentrations of squalene, the sterol precursor in all sterol biosynthetic pathways, differed significantly among *Ochromonas* sp. of different nutritional modes (ANOVA, $p < 0.001$, $n = 8$). Although sterol absolute concentrations in mixotrophs were generally lower than in autotrophs and heterotrophs, relative amounts did not differ among nutritional modes (Table 2). Stigmasterol accounted for up to 98% of the total sterol pool in all flagellates. In contrast, the bacterial diet presented sitosterol and cholesterol as the predominant sterols (Table 2). Two significant roots were extracted from the DA (Fig. 3) run for comparing sterol absolute concentrations among *Ochromonas* sp. of different nutritional modes (*Wilk's Lambda* = 0.151, $F_{10,30} = 4.73$, $p < 0.001$). The first root separated autotrophs from the other nutritional modes because of differences in cholesterol and sitosterol concentrations. The second root separated heterotrophs from mixotrophs because of differences in campesterol and lathosterol concentrations.

Grazing and growth rates—Heterotrophically grown *Ochromonas* sp. showed grazing rates on bacteria (4.16 ± 0.58 cells flagellate^{-1} h^{-1}) that were almost twice as high as those of mixotrophic *Ochromonas* sp. (2.27 ± 0.20 cells flagellate^{-1} h^{-1}) (Mann–Whitney *U*-test, $p < 0.01$, $n = 4$). The growth rate (mean ± SD) of mixotrophic *Ochromonas* sp. was significantly higher than that of heterotrophic flagellates (0.48 ± 0.02 and 0.30 ± 0.02 d^{-1}, respectively; Mann–Whitney *U*-test, $p < 0.01$, $n = 4$). The growth rate of autotrophic *Ochromonas* sp. was 0.07 ± 0.07 d^{-1} (Tittel et al. 2003).

To certify that cells growing mixotrophically and autotrophically did differ from those growing heterotrophically, we measured and compared the cell volume of 60 *Ochromonas* cells of each nutritional mode. Usually, this differentiation is made by microscopically comparing cells and looking for the presence of green chloroplasts. Unfortunately this technique is not applicable to *Ochromonas* sp. because of its natural brown color. We assume that autotrophically and mixotrophically growing cells should be larger than those growing heterotrophically because of the presence of a higher number of intracellular structures involved in photosynthesis, such as chloroplasts. Indeed, cell volume of mixotrophic (52.4 ± 24.1 μm^3) and autotrophic *Ochromonas* (53.0 ± 18.4 μm^3) was similar to (ANOVA, $p > 0.05$, $n = 60$), but significantly larger than,

Table 1. Fatty acid composition of *Ochromonas* sp. cultured autotrophically, mixotrophically, or heterotrophically, as well as of the bacterial diet of mixotrophic and heterotrophic *Ochromonas* sp.

	Ochromonas sp.			
Fatty acid	Autotroph (μg mg C^{-1}) (SD)	Mixotroph (μg mg C^{-1}) (SD)	Heterotroph (μg mg C^{-1}) (SD)	Bacterial diet (μg mg C^{-1}) (SD)
14:0	0.44 (0.21)	0.70 (0.29)	0.31 (0.16)	0.15 (0.02)
15:0	0.86 (0.51)	2.20 (0.41)	0.31 (0.22)	0.09 (0.01)
16:0	14.04 (4.09)	28.47 (20.13)	2.22 (0.53)	1.28 (0.12)
17:0	3.57 (1.51)	5.66 (1.93)	0.13 (0.03)	0.10 (0.05)
18:0	1.57 (0.72)	14.52 (8.47)	1.89 (0.64)	0.51 (0.05)
19:0	—	1.82 (0.78)	0.04 (0.01)	—
20:0	1.96 (1.05)	3.50 (2.05)	—	—
24:0	5.39 (0.24)	5.41 (3.67)	3.45 (1.47)	1.34 (0.23)
Sum SAFA	37.96 (13.95)	89.38 (5.95)	10.82 (4.13)	3.71 (0.12)
14:1ω7	0.44 (0.17)	0.19 (0.10)	0.14 (0.05)	0.04 (0.01)
14:1ω5	0.66 (0.26)	0.52 (0.26)	0.65 (0.17)	0.29 (0.03)
15:1	0.54 (0.32)	14.22 (5.92)	0.24 (0.08)	0.10 (0.01)
16:1ω9	0.86 (0.63)	—	0.05 (0.03)	0.03 (0.01)
16:1ω5	0.81 (0.31)	0.54 (0.30)	0.05 (0.02)	0.08 (0.02)
17:1	7.88 (1.77)	6.37 (2.46)	1.39 (0.39)	0.82 (0.15)
18:1ω9	1.47 (0.79)	0.79 (0.32)	1.04 (0.74)	3.72 (0.99)
18:1ω7	2.07 (0.91)	0.29 (0.23)	0.68 (0.20)	0.39 (0.07)
20:1ω9	1.45 (0.98)	—	—	—
20:1ω11	0.72 (0.41)	0.99 (0.18)	—	—
22:1ω9	6.60 (2.40)	6.58 (2.48)	—	—
Sum MUFA	40.58 (19.97)	46.08 (15.67)	6.89 (4.41)	5.74 (1.20)
16:2ω4	2.17 (1.00)	2.11 (0.50)	0.12 (0.05)	0.32 (0.14)
16:3ω4	1.55 (0.41)	0.92 (0.47)	0.31 (0.12)	0.25 (0.01)
18:2ω6	1.89 (0.59)	1.24 (1.02)	0.73 (0.34)	0.39 (0.07)
18:3ω6	1.91 (0.43)	0.55 (0.25)	0.20 (0.08)	0.14 (0.05)
18:3ω4	—	0.74 (0.20)	—	—
18:3ω3	1.20 (0.64)	0.54 (0.16)	0.20 (0.12)	0.15 (0.09)
18:4ω3	3.96 (1.52)	0.20 (0.12)	0.90 (0.22)	0.45 (0.09)
20:2ω6	2.60 (1.80)	5.16 (1.86)	—	—
20:3ω6	5.19 (3.52)	5.57 (2.68)	—	—
20:4ω6	1.70 (1.39)	—	—	—
20:3ω3	0.41 (0.13)	1.69 (1.06)	—	—
20:4ω3	4.40 (1.95)	—	—	—
20:5ω3 (EPA)	1.62 (0.85)	6.77 (1.20)	—	—
21:5ω3	11.34 (4.78)	4.08 (2.44)	—	—
22:5ω3 (DPA)	2.49 (0.17)	—	—	—
22:6ω3 (DHA)	6.00 (0.32)	7.35 (0.14)	—	—
Sum PUFA	62.05 (28.14)	23.00 (8.05)	3.32 (1.07)	2.00 (0.34)
ω6:ω3	0.4 (0.1)	0.8 (0.2)	0.7 (0.1)	0.9 (0.2)

Sum of SAFA, MUFA, and PUFA takes all measured fatty acids into account, i.e., including those not shown; Values are given as absolute concentrations (μg mg C^{-1}); SD values in parentheses; n = 8; — not detected. DPA: docosapentaenoic acid.

that of heterotrophic *Ochromonas* (38.1 ± 18.1 μm^3; ANOVA, $p < 0.05$, $n = 60$).

Discussion

Biochemistry and trophic mode—The fatty acid profile of *Ochromonas* sp. reflected its nutritional mode, with decreasing concentrations of PUFAs from autotrophic via mixotrophic to heterotrophic flagellates. Interestingly, the same tendency was found for the flagellated chlorophyte *Chlamydomonas* sp., which shows autotrophy as the main nutritional mode in situ (Heifetz et al. 2000; Poerschmann et al. 2004). Together with *Ochromonas* sp., which is mainly heterotrophic in situ, *Chlamydomonas* sp. dominates the plankton community in L111 (Poerschmann et al. 2004). Regardless of the protist species, the tendency of decreasing PUFA concentrations with increasing phagotrophy probably represents a biochemical response to the metabolic adaptations imposed on organisms that change their growth pathway (Hochachka and Somero 2002). Thus, given that mostly saturated bacterial fatty acids make up a large fraction of the fatty acid pool, the higher percentages of SAFAs in mixotrophs and heterotrophs than in autotrophs probably result from phagotrophy. Also, the lower total content of all fatty acid classes in heterotrophs compared with mixotrophs despite the higher bacterial ingestion rates by heterotrophs may be a sign of changes in extrachloroplastic membranes as well as loss of chloroplast lipids (Lösel 1988), since loss of chlorophyll occurs with increased phagotrophy (Caron et al. 1990; Sanders et al. 1990).

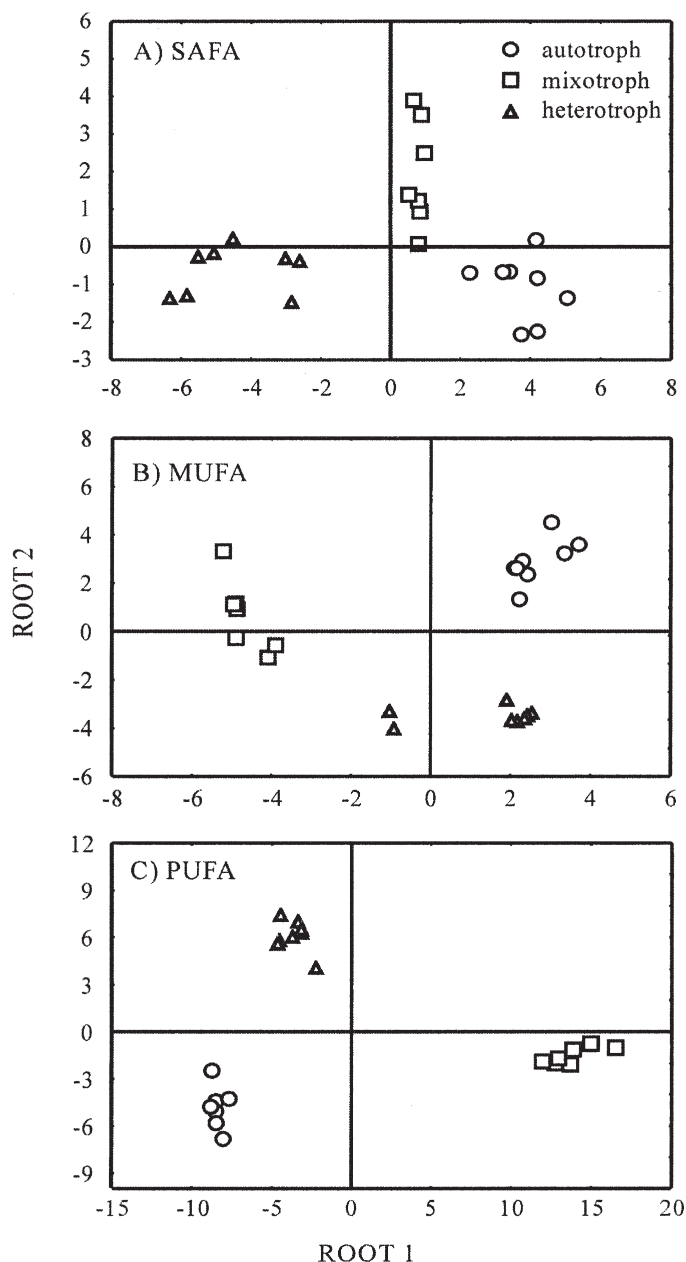

Fig. 2. Canonical roots provided by DAs performed for (A) saturated (SAFA), (B) monounsaturated (MUFA), and (C) polyunsaturated (PUFA) fatty acids of autotrophic (open circles), mixotrophic (open triangles), and heterotrophic (open squares) *Ochromonas* sp. *See text and Table 1* for single fatty acids discriminating within a root.

There is a debate about the ecological advantages of mixotrophy. Some authors argue that additional phagotrophy is a means of acquiring nutrients for photosynthesis during periods of nutrient limitation (Nygaard and Tobiesen 1993). Other authors suggest that the prime purpose of mixotrophy is to obtain carbon for growth (Caron et al. 1990; Jones et al. 1993). If so, carbon obtained by phagotrophy may be primarily used for building structural biomolecules such as polysaccharides, whereas photosynthesis is responsible for generating substrates and energy for the synthesis of more complex biomolecules such as PUFAs. According to our DAs, long-chain PUFAs were a better indicator of the nutritional mode of *Ochromonas* sp. than MUFAs or SAFAs. This is not surprising since energetic costs of building PUFAs are much higher than those for MUFAs or SAFAs. Also, the presence of a broader number of accessory enzymatic systems is necessary to insert double bonds into the hydrocarbon chain (Ratledge and Wilkinson 1988; Berg et al. 2002). The DA also identified single PUFAs, accounting for the best separation of *Ochromonas* sp. grown under different nutritional pathways. Here, the first canonical root of the DA run for PUFAs showed that fatty acids belonging to the $\omega3$ family (e.g., 18:3ω3, 20:3ω3) were most effective in distinguishing mixotrophs from autotrophs. Furthermore, the absence of long-chain fatty acids separated heterotrophs at the second root of the DA, pointing to an inability of heterotrophs to elongate hydrocarbon chains of fatty acids or even to an energetic constraint of the heterotrophic life strategy, which could have contributed to the overall lower total contents of SAFAs, MUFAs, and PUFAs in heterotrophs compared with mixotrophs.

A predominance of stigmasterol in the sterol profile of *Ochromonas* sp. has already been described for several species of the genus, such as *Ochromonas danica* (Halevy et al. 1966), *Ochromonas malhamensis* (Avivi et al. 1967), and *Ochromonas sociabilis* (Goodwin 1974). Interestingly, except for stigmasterol, the sterol composition of mixotrophs resembled that of bacteria but differed from those of autotrophs and heterotrophs, whose sterol compositions were quite similar. Possibly, mixotrophy slows down sterol synthesis, leading to sterol shortage and reduced sterol storage. That mixotrophs use both autotrophic and heterotrophic metabolisms implies higher metabolic costs compared with more specialized organisms such as heterotrophs and autotrophs (Rothhaupt 1996*a*). Moreover, as mixotrophs use two metabolisms, there may be "cross-talk" between the biosynthetic pathways resulting in the synthesis of compounds not found in heterotrophs and autotrophs. In mixotrophs, sterol synthesis may have been concentrated on stigmasterol synthesis, since stigmasterol concentrations of mixotrophs were similar to those of autotrophs and heterotrophs, while concentrations of other sterols were lower in mixotrophs. Thus, the synthesis of other sterols may have been reduced to a minimum necessary to guarantee cellular activity. This hypothesis is supported by the finding that, despite differences in absolute sterol concentrations, sterol percentages remained similar regardless of nutritional mode. According to the DA, differences in cholesterol distinguish autotrophs from heterotrophs, whereas the phytosterol sitosterol allows autotrophs to be differentiated from mixotrophs. Cholesterol synthesis requires molecular oxygen and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), and it is thus supposed to be regulated by photosynthesis and respiration rates (Berg et al. 2002). On the other hand, the synthesis of phytosterols is regulated by the presence of precursors that differ from those needed for cholesterol synthesis (Moreau et al. 2002). The presence of phytosterol precursors may have accounted for the differences in

Table 2. Sterols and sterol precursor (squalene) in *Ochromonas* sp. cultured autotrophically, mixotrophically, or heterotrophically as well as of the bacterial diet of mixotrophic and heterotrophic *Ochromonas* sp.

Sterol	*Ochromonas* sp. Autotroph	Mixotroph	Heterotroph	Bacterial diet
Cholesterol (μg mg C^{-1})	3.8 (2.5)	0.1 (0.01)	1.6 (0.2)	0.4 (0.2)
%	**6.1 (1.4)**	**1.3 (0.1)**	**3.6 (1.3)**	**8.9 (1.3)**
Campesterol (μg mg C^{-1})	—	0.03 (0.01)	0.4 (0.2)	0.04 (0.04)
%		**0.3 (0.1)**	**0.7 (0.2)**	**3.0 (2.3)**
Stigmasterol (μg mg C^{-1})	38.9 (18.7)	22.8 (7.4)	39.9 (9.2)	0.1 (0.1)
%	**91.9 (3.3)**	**97.7 (2.3)**	**93.7 (1.8)**	**7.2 (3.4)**
Sitosterol (μg mg C^{-1})	2.1 (1.5)	0.2 (0.03)	0.9 (0.3)	1.1 (1.0)
%	**2.7 (0.6)**	**1.6 (0.1)**	**2.1 (0.6)**	**80.5 (3.5)**
Lathosterol (μg mg C^{-1})	0.2 (0.1)	0.02 (0.01)	0.2 (0.1)	0.02 (0.01)
%	**0.3 (0.1)**	**0.2 (0.1)**	**0.3 (0.1)**	**2.7 (2.5)**
Unidentified* (%)	**1.5 (0.1)**	—	—	—
Total (μg mg C^{-1})	62.4 (41.0)	22.9 (7.4)	48.7 (17.3)	1.6 (1.2)
Squalene (μg mg C^{-1})	6.1 (1.1)	1.6 (1.1)	2.2 (0.4)	0.7 (0.2)

Values are given as absolute concentrations in μg mg C^{-1} ($\pm$SD; $n = 8$) and percentages ($\pm$SD; $n = 8$) of the total sterol pool (values in bold); — not detected.
* Quantitative analysis only possible as percentage of the total neutral lipid fraction.

sitosterol concentrations between autotrophs and mixotrophs. Taken together, sterols were by far less efficient to distinguish between nutritional modes of *Ochromonas* sp. than PUFAs were, suggesting that the nutritional mode of this flagellate may be linked more tightly to the metabolism of PUFAs than to that of sterols.

Mixotrophy as an ecological strategy—Our results show that *Ochromonas* sp., which is predominantly heterotrophic in situ (Fenchel 1982; Rothhaupt 1996*b*), carried out photosynthesis in addition to grazing bacteria, suggesting that bacteria were not abundant or nutritive enough to support optimal cellular growth and reproduction. In other words, under limiting conditions, *Ochromonas* sp. carried out both strategies, which was reflected by its characteristic fatty acid composition. Mixotrophs contained high concentrations of SAFAs, as a result of bacterivory, but they also contained PUFAs that were found to be typical for autotrophs. Hence, carbon uptake and metabolism is most likely the primary factor driving mixotrophy in *Ochromonas* sp., and the metabolic patterns of PUFA synthesis and allocation seem to be regulated by the availability of light. Similarly, the polyunsaturated fatty acid profiles of *Chlamydomonas* sp., which is mainly found as a phototroph in situ, were dictated by light when this alga was cultured either autotrophically, mixotrophically, or heterotrophically (Poerschmann et al. 2004). The enzyme acetyl CoA carboxylase catalyzes the first step of fatty acid synthesis, and its activity depends on both light and the rate of lipid synthesis in the chloroplast (Post-Beitenmiller et al. 1992). De novo synthesis of lipids in plants also depends on NADPH produced by the light reactions of photosynthesis. Hence, lipid synthesis and the allocation of photosynthates into lipids are both supposed to increase in the presence of light (Wainman and Lean 1992), regardless of the trophic strategy adopted by the protist in situ.

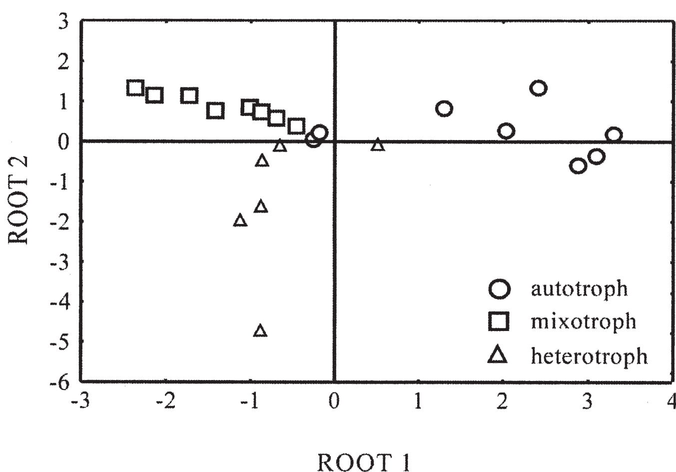

Fig. 3. Canonical roots provided by DAs performed for the sterol composition of autotrophic (open circles), mixotrophic (open triangles), and heterotrophic (open squares) *Ochromonas* sp. *See text and Table 2* for single sterols discriminating within a root.

Currently accepted resource competition theory (rct) predicts that trophic specialization (autotrophy, heterotrophy) should be the most successful strategy under nonlimiting conditions because specialists do not bear the metabolic costs of running both an autotrophic and a heterotrophic system. However, mixotrophy should represent a competitive advantage for organisms growing under limiting conditions when the advantages of having access to both of the resources light and organic matter outweigh the metabolic costs of running both trophic systems (Rothhaupt 1996*a*; Jones 2000). The rct implies that light and organic matter are substitutable resources for mixotrophic organisms. In the case of the predominantly bacterivorous mixotroph *Ochromonas,* rct predicts that additional photosynthesis in mixotrophic flagellates leads to an increase in growth rate under limiting conditions and that the growth rate of mixotrophs amounts to the sum of the production that autotrophic and bacterivorous specialists realize at the same resource availability and resource use efficiency minus the metabolic costs of running both trophic systems. Therefore, the theoretical growth rate of mixotrophs (r^h_{mix}) according to rct can be

roughly estimated based on the data presented in this study:

$$r^h_{mix} = \frac{G_{mix}}{G_{bact}} r_{bact} + r_{aut} \quad (5)$$

where G_{mix} is the grazing rate of mixotrophs, G_{bact} is the grazing rate of strict bacterivores growing at the same bacterial density as mixotrophs, r_{bact} is the production of strict bacterivores, and r_{aut} is the production of strict autotrophs growing under the same light conditions as mixotrophs. Interestingly, r^h_{mix} estimated as described above amounts to 0.23 ± 0.08 d^{-1} (mean $\pm$ SD) and is thus much lower than the growth rate of mixotrophs in our experiments (0.48 ± 0.02 d^{-1}). Hence, there are strong synergetic effects of the combination of heterotrophic and autotrophic mode that cannot be explained by classical rct. Our results on the biochemistry of *Ochromonas* suggest that these synergetic effects may result from the combination of the advantages of phototrophic and heterotrophic metabolic pathways to synthesize complex and essential biochemical compounds. Moreover, significantly lower grazing rates of mixotrophic flagellates than those of heterotrophic flagellates suggest that mixotrophs regulate the proportions of heterotrophic and autotrophic metabolism and consequently also the synergetic effects of combined metabolic and biochemical possibilities in order to achieve a trade-off for maximal growth. It is important to note that the nonsignificant growth rates of autotrophically growing *Ochromonas* (0.07 ± 0.07 d^{-1}) may be a result of combined insufficient phototrophic abilities and insufficient bacterial prey densities in the cultures. Possibly, light may only contribute to *Ochromonas* growth when a certain bacterial density is available.

Our results may also challenge the notion that mixotrophy puts an organism at a competitive disadvantage with autotrophs or heterotrophs under nonlimiting conditions because synergetic effects arising from the combination of heterotrophic and autotrophic metabolism may outweigh the higher metabolic costs of maintaining both trophic systems. This could be tested by determining growth efficiency and the biochemical composition of nonlimiting grown *Ochromonas*.

Implications for aquatic food webs—Knowledge on the metabolic consequences of different trophic strategies may help ecologists to evaluate trophic interactions and the flow of biochemical matter through aquatic food webs. For instance, the adoption of different trophic strategies in protist species that could compete within the same habitat of an ecosystem has been regarded as a strategy that allows for a trophic and thus spatial separation of these species. This is the case of *Ochromonas* sp. and *Chlamydomonas* sp., both living in the acidic L111 in East Germany. *Ochromonas* sp. is the dominant species in the epilimnion of L111, whereas *Chlamydomonas* sp. dominates the community in the deep chlorophyll maximum (DCM) (Tittel et al. 2003; Kamjunke et al. 2004). However, such a spatial separation of trophic strategies should lead to a pronounced spatial heterogeneity in the nutritional quality of available food in this ecosystem.

From the autecological perspective, the fact that one nutritional strategy may predominate over others within the dominant protist species of extreme ecosystems, such as mixotrophy in *Pyramimonas gelidicola* living in Antarctic lakes (Bell and Laybourn-Parry 2003) or heterotrophy in *Ochromonas* sp. and autotrophy in *Chlamydomonas* sp., both species living in acidic mining lakes (Tittel et al. 2003), may impose certain nutritional constraints on predators in such ecosystems. For instance, the absence of PUFAs in heterotrophic *Ochromonas* sp. may limit its nutritional quality as prey, since some PUFAs are recognized to be essential for zooplankton species (Müller-Navarra et al. 2004). Since prey availability is often temporally or spatially limited in extreme environments (Weithoff 2004), the nutritional quality of available prey may affect or even determine the fluxes of biochemical matter and energy through food webs, and thus also affect community structure and productivity of these ecosystems.

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