Osmotrophic glucose and leucine assimilation and its impact on EPA and DHA content in algae

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The uptake of dissolved organic compounds, i.e. osmotrophy, has been shown to be an efficient nutritional strategy for algae. However, this mode of nutrition may affect the biochemical composition, e.g. the fatty acid contents, of algal cells. This study focused on the osmotrophic assimilation of glucose and leucine by selected seven algal strains belonging to chlorophytes, chrysophytes, cryptophytes, dinoflagellates and euglenoids. Our laboratory experiments with stable isotope labeling showed that osmotrophy occurred in four of the selected seven strains. However, only three of these produced long chain omega-3 fatty acids eicosapentaenoic acid (EPA; 20:5ω3) and docosahexaenoic acid (DHA; 22:6ω3). High glucose content (5 mg L^{-1}) affected negatively on the total fatty acids of *Mallomonas kalinae* and the total omega-3 fatty acids of *Cryptomonas* sp. Further, glucose assimilation explained 35% (negative effect) and leucine assimilation 48% (positive effect) of the variation of EPA, DHA and the fatty acids related to their synthesis in *Cryptomonas* sp. Moderate glucose concentration (2 mg L^{-1}) was found to enhance the growth of *Cryptomonas ozolinii*, whereas low leucine (20 µg L^{-1}) enhanced the growth of *Mallomonas kalinae*. However, no systematic effect of osmotrophy on growth rates was detected. Our study shows that osmotrophic assimilation of algae is species and compound specific, and that the effects of the assimilated compounds on algal metabolism also varies depending on the species.

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Abstract

The uptake of dissolved organic compounds, i.e. osmotrophy, has been shown to be an efficient nutritional strategy for algae. However, this mode of nutrition may affect the biochemical composition, e.g. the fatty acid contents, of algal cells. This study focused on the osmotrophic assimilation of glucose and leucine by selected seven algal strains belonging to chlorophytes, chrysophytes, cryptophytes, dinoflagellates and euglenoids. Our laboratory experiments with stable isotope labeling showed that osmotrophy occurred in four of the selected seven strains. However, only three of these produced long chain omega-3 fatty acids eicosapentaenoic acid (EPA; 20:5ω3) and docosahexaenoic acid (DHA; 22:6ω3). High glucose content (5 mg L^{-1}) affected negatively on the total fatty acids of *Mallomonas kalinae* and the total omega-3 fatty acids of *Cryptomonas* sp. Further, glucose assimilation explained 35% (negative effect) and leucine assimilation 48% (positive effect) of the variation of EPA, DHA and the fatty acids related to their synthesis in *Cryptomonas* sp. Moderate glucose concentration (2 mg L^{-1}) was found to enhance the growth of *Cryptomonas ozolinii*, whereas low leucine (20 µg L^{-1}) enhanced the growth of *Mallomonas kalinae*. However, no systematic effect of osmotrophy on growth rates was detected. Our study shows that osmotrophic assimilation of algae is species and compound specific, and that the effects of the assimilated compounds on algal metabolism also varies depending on the species.

Introduction

Mixotrophy, i.e. the ability of an organism to combine autotrophy and heterotrophy and thus get sustenance simultaneously from inorganic and organic sources, is gaining increasing attention in studies of aquatic as well as terrestrial ecosystems. In aquatic habitats, mixotrophy is common in unicellular organisms such as algae, cyanobacteria and protists (Flynn et al. 2013; Schmidt et al. 2013). The heterotrophic nutrition in mixotrophy is via phagotrophy (particle or cell uptake) or osmotrophy (uptake of dissolved organic compounds), both of which may occur in tandem with photosynthesis or during dark periods, e.g. nights.

The detection of mixotrophic behavior can be challenging both in laboratory and field conditions, and thus, for example, radioactive isotope labelling has been applied to study the osmotrophic nutrient uptake (Kamjunke & Tittel 2008; Tittel et al. 2009; Beamud et al. 2014). These studies have shown that many algae have the ability to assimilate carbohydrates (e.g. glucose), amino acids (e.g. glutamine, leucine, thymidine, aspartic acid) and other organic compounds (e.g. acetic acid, coumaric acid, glycerol), which they use as carbon and nitrogen sources, and which are commonly released by the algae themselves or ~~e.g.~~ by bacteria (Hellebust 1965; Kamjunke& Tittel 2008; Tittel et al. 2009; Beamud et al. 2014; Dąbrowska et al. 2014). Osmotrophy has been shown to be an efficient nutrition strategy for algae in nature: osmotrophic assimilation of amino acids prevent nitrogen limitation, which favors biomass growth in oligotrophic lakes (Kamjunke & Tittel 2008). Similarly, osmotrophic uptake of fulvic acids enhances biomass growth and boost bloom forming in humic lakes (Rengefors et al. 2008).

Osmotrophic nutrition may affect the biochemical composition of algal cells. Biosynthesis of various molecules is determined by phylogeny-based traits and there is a significantly different composition of, for example, fatty acids (FAs) in different algal taxa (Kohli et al. 2016). It is reported that growth conditions account for relatively low variation in algal FAs compared to phylogeny (Galloway & Winder 2015), however, studies with *Ochromonas* sp. (Boechat et al. 2007) showed decreased polyunsaturated fatty acid (PUFA) concentration by feeding mode. Thus, even though the FA profiles and the quality of synthetized FAs may not change, the quantity of different FAs might be affected by the growth mode. For example, nitrogen limitation favors FA synthesis and lipid accumulation in algal cells, and thus if algae can assimilate leucine

and use it as their nitrogen source, they should not start to accumulate lipids but carry on cell division as long as ~~also~~ the other essential nutrients are available. In turn, osmotrophically assimilated glucose is channeled directly into lipid synthesis, i.e. to palmitic acid (16:0), which results in building up of triacylglycerols and e.g. long chain polyunsaturated fatty acids (LC-PUFAs; Ratledge 2004). Some LC-PUFAs belong to omega-3 FAs (e.g. eicosapentaenoic acid [EPA; 20:5ω3] and docosahexaenoic acid [DHA; 22:6ω3]), and for their part are of utmost importance for the growth and reproduction of consumers in aquatic food webs (Peltomaa et al. 2017; Taipale et al. 2018). Since algae are practically the only primary source of EPA and DHA in aquatic food webs (Colombo et al. 2017), osmotrophic nutrient uptake may affect the whole food web by influencing the availability of these nutritionally- essential compounds, and thus the ~~performance, i.e. the~~ growth and reproduction~~,~~ of upper trophic levels (Jonasdottir 1994; Brett et al. 2006; Peltomaa et al. 2017; Taipale et al. 2018).

In this study we focused on the osmotrophic uptake of glucose and leucine by selected seven algal strains belonging to chlorophytes, chrysophytes, cryptophytes, dinoflagellates and euglenoids. We conducted short-term stabile isotope labelling experiments with these algae to determine if they are able to assimilate glucose and/or leucine. Since we were especially interested in the effects of osmotrophy on EPA and DHA production, we analyzed the fatty acids of these strains. For studying the impact of osmotrophy on fatty acid synthesis, we selected ~~the~~ EPA and DHA synthesizing osmotrophic strains and cultured them with glucose, leucine and mix of these two in a long-term experiment for 14 days, i.e. until the cultures reached the stationary or late exponential phase, during which the LC-PUFAs are mobilized from the membranes into storage lipids (Roessler 1990; Boelen et al. 2017). We hypothesized that (1) all of the strains are osmotrophic, i.e. assimilate glucose and leucine, (2) osmotrophy has positive effect on their growth, and that (3) the osmotrophic uptake of glucose increases the EPA and DHA concentrations in algae capable of synthesizing these fatty acids, whereas (4) the uptake of leucine does not affect specifically their EPA and DHA concentrations, but may actually lower their total FA content.

Materials & Methods

The algal strains and growth conditions

The studied algal strains were from freshwater origin, and included chlorophytes *Chlamydomonas reinhardtii* (from the collection of the University of Washington, UWCC) and *Selenastum* sp. (SCCAP K-1877), chrysophyte *Mallomonas kalinae* (SCCAP K-1759), cryptophytes *Cryptomonas* sp. (CPCC 336) and *C. ozolinii* (UTEX LB 2782), dinoflagellate *Peridinium* sp. (author's collection, isolated from Lake Valkea-Kotinen, Finland, 61.14°N, give longitude as well? in 2015) and euglenoid *Euglena gracilis* (CCAP 1224/5Z). The stock cultures of the strains were grown autotrophically in AF6 medium (*E. gracilis*; Watanabe 2000) or MWC medium (all the other strains; Guillard & Lorenzen 1972) at 20 °C under light:dark cycle of 16:8 h with light intensity of 70-100 µmol m^{-2} s^{-1}. The cultures were not axenic, but the initial numbers of bacteria were low due to the growth media consisting of inorganic nutrients. The bacterial numbers were not determined, but bacterial FA biomarkers were included in the FA analysis (see below). The algal cultures were grown to late exponential phase before they were used in the experiments.

Short-term stable isotope labelling experiments

For the short-term stable isotope experiment, the autotrophically grown seven algal strains were grown to stationary phase, collected with centrifugation into pellets (200 mL, 5 min, 2000 rpm, which was pre-examined as safe for the fragile flagellates), and further resuspended into 50 mL of fresh AF6 (*E. gracilis*) or MWC media (all the other strains) enriched with glucose 5 mg L^{-1} (containing 4.9 mg L^{-1} non-labeled D-(+)-Glucose [Sigma-Aldrich Co, St Louis, MO, USA] and 0.1 mg L^{-1} ^{13}C-labeled D-Glucose [Sigma-Aldrich Co.]) or with leucine 400 µg L^{-1} (containing 392 µg non-labeled L-Leucine [Sigma-Aldrich Co.] and 8 µg ^{15}N-labeled L-Leucine [Sigma-Aldrich Co.]) or with both. Three independent replicates were used for each algae and treatment, and non-labeled autotrophic controls were run in parallel. The incubation took place at 20 °C under a constant light intensity of 70-100 µmol m^{-2} s^{-1}, i.e. the possible dark-time heterotrophic assimilation of glucose and leucine was excluded from this short-term experiment. The incubation time was only 30 minutes to preventing respiration loss of the labels, but it was still long enough to acquiring detectable changes in cellular δ^{13}C and δ^{15}N concentrations. After the incubation, the samples were centrifuged (2000 rpm, 5 min), the supernatants were discarded, and the pellets were flushed by diluting them into 30 mL of fresh AF6 (*E. gracilis*) or MWC media (all the other strains) and centrifuging them again. After discarding the supernatants, the

pellets were frozen in -80 °C, and freeze-dried within two days. The $\delta^{13}C$ and $\delta^{15}N$ as well as fatty acid profiles were analyzed from these samples.

The $\delta^{13}C$ and $\delta^{15}N$ analyses

For the $\delta^{13}C$ and $\delta^{15}N$ analyses, approximately 2 mg of the freeze-dried algal biomass was weighed into tin capsules. The analyses were carried out on a Carlo-Erba Flash 1112 series Element Analyzer connected to a Thermo Finnigan Delta Plus Advantage IRMS (Thermo Fisher Scientific, USA). Four replicates were run from each sample. The samples were compared to the NBS-22 standard using birch leaf powder as a laboratory-working standard. The precision of the $\delta^{13}C$ and the $\delta^{15}N$ analyses were 0.2% and 0.3%, respectively, for all samples.

Fatty acid analysis

Two replicates of each freeze-dried sample were weighed (1-2 mg/sample) into tin capsules and the lipids were extracted using chloroform:methanol (2:1) NaCl -method (Parrish 1999). Toluene and sulfuric acid were used for the transesterification of fatty acid methyl esters (FAMEs) at 90 °C for 1 h. The FAMEs were analyzed with a gas chromatograph (Shimadzu Ultra, Japan) equipped with a mass detector (GC-MS; Shimadzu Ultra, Kyoto, Japan) and using helium as a carrier gas and an Agilent® (Santa Clara, CA, USA) DB-23 column (30 m × 0.25 mm × 0.15 μm). Fatty acid concentrations were calculated using calibration curves based on known standard solutions of a FAME standard mixture (GLC standard mixture 566c, Nu-ChekPrep, Elysian, MN, USA) (see Taipale et al. 2016 for further details). The 16:0, alpha-linolenic acid (ALA; 18:3ω3), stearidonic acid (SDA, 18:4ω-3), EPA and DHA concentrations, and the total sum of monounsaturated fatty acids with 16 carbons (16 MUFAs) and 18 carbons (18 MUFAs) were in the focus of this study and thus reported here. The fatty acid biomarkers for bacteria (i-14:0, i-15:0, a-15:0, i-16:0, i-17:0 and a-17:0; Brennan 1989; Taipale et al. 2015) were also detected in order to ensure that the numbers of bacteria were low, i.e. the glucose and leucine were assimilated by the algae, not by bacteria.

The long-term osmotrophy experiments

The long-term experiments were done only for those three strains that were detected to be osmotrophic (i.e. assimilated either glucose or leucine or both in the short-term experiment) and,

based on the results from the short-term study, were detected to synthetize either EPA or DHA or both. These strains were: *M. kalinae*, *Cryptomonas* sp. and *C. ozolinii*. The long-term experiments were run in independent triplicates, and using three different glucose (0.5, 2 and 5 mg L^{-1}) and leucine (20, 100 and 400 µg L^{-1}) concentrations selected based on literature (Kamjunke et al. 2008, Kamjunke & Tittel 2008). There were no mixed treatments of glucose and leucine, but autotrophic controls were run in parallel. The algal cells were collected into pellets from the stock cultures similarly to short-term experiments before transferring them into the experimental flasks of 250 mL. The strains were grown for 15-16 days in similar conditions as the algal stock cultures and the growth was followed through microscopic counts every third day using Sedgewick Rafter –counting cells and preservation with acid Lugol's solution (Willén 1962). The specific growth rates (µ; d^{-1}) for all strains were calculated using the equation 1. The cells were grown into the stationary or late exponential phase (Fig. S1), harvested during the light-period of the light:dark-cycle, and pelleted, frozen (-80 °C) and freeze-dried. The fatty acid profiles were analyzed similarly to the short-term samples (see above), but only from two replicates of each treatment and from one control.

$$\mu = \ln(cellsTx/cellsT0)/(Tx-T0) \quad (1)$$

where:

µ is the specific growth rate

cells T0 is the cell number at time 0 (T0)

cells Tx is the cell number at time x (Tx)

Statistical analyses

The results of the short-term isotope labelling experiments were statistically tested with t-test by comparing the non-labeled autotrophic control samples with the labeled samples. The effects of osmotrophy on growth and FA contents were in the long-term experiment tested with the analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) post hoc test. Levene's test was used for testing the homogeneity of variances. Principal component analysis (PCA), permutational multivariate analysis of variance (PERMANOVA) and similarity percentages (SIMPER) were used for a more detailed study of the similarity of the FA profiles

between the treatments in the long-term experiment. In PCA the 16:0, alpha-linolenic acid (ALA; 18:3ω3), stearidonic acid (SDA, 18:4ω-3), EPA and DHA concentrations, and the total sum of monounsaturated fatty acids with 16 carbons (16 MUFAs) were included in the analysis. In PERMANOVA and SIMPER the analysis included the above mentioned FAs and also MUFAs with 18 carbons (18 MUFAs). All glucose treatments and all leucine treatments were pooled for the analysis in PERMANOVA and SIMPER. PERMANOVA was run with unrestricted permutation of raw data and type III sums of squares. Monte Carlo adjustment was used in PERMANOVA due to low ~~amount~~ numbers of replicates. In the statistical testing, p-values < 0.05 were considered as significant. ANOVA and Tukey's and Levene's tests were conducted with IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY, USA). PCA, PERMANOVA and SIMPER were done using Primer 7 (version 7.0.13, Quest Research Limited, Auckland, New Zealand).

Results

The osmotrophic glucose and leucine uptake

The studied strains showed different responses to glucose and leucine additions in the short-term experiment (Fig. 1, Table S1). The chlorophyte *C. reinhardtii,* the dinoflagellate *Peridinium* sp. and the euglenoid *E. gracilis* did not show glucose or leucine assimilation at all. Whereas, the chlorophyte *Selenastrum* sp. assimilated both compounds (glucose t-test $p < 0.001$, leucine t-test $p = 0.04$), as did also the cryptophytes *Cryptomonas* sp. (glucose t-test $p < 0.01$, leucine t-test $p = 0.02$) and *C. ozolinii* (glucose t-test $p < 0.01$, leucine t-test $p < 0.001$). The chrysophyte *M. kalinae* did not assimilate glucose, but assimilated leucine (t-test $p < 0.01$).

Fatty acid profiling of the short-term experiment

The fatty acid profiling of the short-term experiments showed that the strains capable of EPA and/or DHA synthesis were *M. kalinae* (average EPA 0.3 % of all FAs, sd 0.0; average DHA 3.2 % of all FAs, sd 0.1), *Cryptomonas* sp. (EPA 17.6 %, sd 0.0; DHA 2.3 %, sd 0.0), *C. ozolinii* (EPA 13.9 %, sd 1.3; DHA 2.1 %, sd 0.2), *Peridinium* sp. (EPA 13.4, sd 0.0; DHA 25.1, sd 0.6) and *E. gracilis* (EPA 14.3 %, sd 0.8; DHA 8.4 %, sd 0.2). However, because only *M. kalinae*, *Cryptomonas* sp. and *C. ozolinii* were showing osmotrophic uptake of glucose and/or leucine,

these three strains were selected for the long-term experiment. Fatty acid biomarkers for bacteria (i-14:0, i-15:0, a-15:0, i-16:0, i-17:0 and a-17:0) were not found in the analysis.

The effect of osmotrophy on growth, total FAs and EPA and DHA production

In general, *C. ozolinii* had higher specific growth rates than the two other studied strains in the long-term experiment. The highest growth rate in *C. ozolinii* (0.88 div. day^{-1}; ANOVA $p < 0.01$) was detected with moderate glucose concentration of 2 mg L^{-1}, and in *M. kalinae* (0.47 div. day^{-1}, ANOVA $p < 0.01$) with low leucine concentration of 20 µg L^{-1}, but otherwise there were no signs that the osmotrophic nutrition would have had increased or decreased the growth rates of the studied three strains (Table 1).

The highest total FA concentration (i.e. 88.8 µg FA in mg DW) was found in the autotrophic *M. kalinae*, and the total fatty acids of *M. kalinae* were also detected to vary according to the treatment that was lowest (33.8 µg FA in mg DW) in the 5 mg L^{-1} glucose treatment (Table 2). However, it could not be firmly stated that either glucose or leucine addition would have had certain effects on the total FA content in *M. kalinae*. There were no treatment-based variation in the total FAs of the two cryptophytes either, but there was species specific variation: the total FA concentrations in *Cryptomonas* sp. (52.8-73.6 µg FA in mg DW) were substantially higher in all treatments than in *C. ozolinii* (range 32.9-45.1 µg FA in mg DW; Table 2). Fatty acid biomarkers for bacteria (i-14:0, i-15:0, a-15:0, i-16:0, i-17:0 and a-17:0) were not found in the FA analysis.

The proportion of omega-3 FAs (i.e. ALA, SDA, EPA and DHA) out of total FAs varied between the strains being lowest in *M. kalinae* (range 21.3-40.3%), and was substantially higher in *Cryptomonas* sp. (range 76.8-89.9%) and in *C. ozolinii* (69.9-94.8%; Fig. 2). The PCA plot of the omega-3 FAs clustered the strains into their own groups in spite of the growth conditions (Fig. 3) indicating strong genetic control of FA profiles and synthesis. When EPA and DHA were studied more in detail, no clear evidence of the effects of osmotrophy on the contents of these FAs were found with ANOVA: in *M. kalinae* EPA and its precursor ALA varied between the treatments, in *Cryptomonas* sp. variations were found in 16:0, ALA and EPA, and in *C. ozolinii* in EPA and DHA, but either glucose or leucine could not be stated to have specific effect on these omega-3 FAs (Tables 2, S2). However, when the 16:0, ALA, SDA, EPA, DHA, 16

MUFA and 18 MUFA concentrations were studied with PERMANOVA, the glucose assimilation were found to explain 35% (PERMANOVA, $F_{(3,6)} = 2.74$, $p = 0.025$) and the leucine assimilation 48% (PERMANOVA, $F_{(3,6)} = 3.78$, $p = 0.021$) of the variation in these FAs in *Cryptomonas* sp. (Table 3). Statistically significant results were not found with PERMANOVA for *M. kalinae* or *C. ozolinii* (Table 3). These observations are in line with the results of the PCA (Fig. 3), indicating that *Cryptomonas* sp. differ from *M. kalinae*, but also from *C. ozolinii*.

Discussion

In this study, we focused on the osmotrophic nutrition and omega-3 FA production of seven algal strains representing chlorophytes, chrysophytes, cryptophytes, dinoflagellates and euglenoids. We expected that all of the studied strains would assimilate glucose and leucine (Hypothesis 1), but this was not the case. The chlorophyte *C. reinhardtii*, the dinoflagellate *Peridinium* sp. and the euglenoid *E. gracilis* did not assimilate either glucose or leucine during the 30 minutes incubation period of our short-time experiment. Furthermore, the chrysophyte *M. kalinae* assimilated only leucine. The uptake velocities of different algae may vary for different compounds (North and Stephens 1972) and depending on the growth mode (Wheeler et al. 1974), and thus it is possible that even though our cultures were on stationary phase and assumingly depleted by nutrients, our 30 min incubation time was not sufficient enough for assimilation for some of the algae used in this study. We did the incubations in light, which also could have affected the results, since in some cases algae have shown higher osmotrophic assimilation in dark than in light for glucose (Beamud et al. 2014) and leucine (Ruiz-González et al. 2012). However, many algal species are reported to enhance their osmotrophic uptake in light (Tittel et al. 2009; Beamud et al. 2014). Additionally, our experiments were done in nutrient-rich AF6 or MWC medium, but inorganic nutrient limitation could have triggered rapid osmotrophic uptake (Kamjunke & Tittel 2008). However, it has also been shown by Beamud et al. (2014) that osmotrophic feeding mode is not triggered only by nutrient deficiency: in their study the chlorophytes *Keratococcus rhaphidioides* and *Watanabea* sp. assimilated leucine, thymidine, aspartic acid and acetate also under high levels of inorganic nitrogen and phosphorus, and not only during nutrient limitation. Altogether, our study and the previous observations show that osmotrophic assimilation of algae is both species and compound specific, and that generalizations on the occurrence of osmotrophy within certain taxa cannot be done.

For studying the effects on osmotrophy on algal fatty acids, especially the LC-PUFAs, the FA profiles of the strains were screened in the short-term study. It is already known that most EPA and DHA producing algae belong to the kingdom Chromista, i.e. to cryptophytes, haptophytes and heterokonts (Cavalier-Smith 2010; Mühlroth et al. 2013). This was the case also with our algae: EPA and DHA was found in all strains excluding the chlorophytes *C. reinhardtii* and *Selenastrum* sp. However, since we specifically focused on the effects of osmotrophic nutrition on EPA and DHA production, only *M. kalinae* and the two cryptophyte strains showing osmotrophic assimilation were studied in our long-term experiment. In this experiment, we expected that (Hypothesis 2) the growth rates would have been enhanced by the osmotrophic growth mode, as has earlier been reported for the raphidophyte *Gonyostomum semen* (Rengefors et al. 2008) and for the chlorophytes *K. rhaphidioides* and *Watanabea* sp. (Beamud et al. 2014). This however was not the case, and excluding some statistically significant differences in the growth rates of *M. kalinae* and *C. ozolinii*, the growth rates could not be directly related to glucose or leucine assimilation. We cannot fully explain the reason behind these two observations on higher growth rates, but are aware that for example mixotrophic phagotrophy on bacteria may increase algal growth (Yoo et al. 2017). We did not observe high amounts of bacteria in the samples during the algal cell counts or detect bacterial FA biomarkers in the FA analysis. However, we did not calculate the bacterial numbers nor studiedy the possible assimilation of bacteria by the algal strains, and thus cannot explicitly state that phagotrophic mixotrophy did not occur during the long-term experiment.

Because glucose is channeled directly to palmitic acid (16:0) and further into lipid synthesis (Ratledge 2004), and the cellular neutral lipid content should be at highest during the light period of the light:dark cycle (Roessler 1990), and because the LC-PUFA content should be at highest during the stationary growth phase (Roessler 1990; Boelen et al. 2017), we expected (Hypothesis 3) that the osmotrophic uptake of glucose increases the FA content in algae. However, we did not find any specific effect of glucose on the amount of total FAs or EPA or DHA. Our PERMANOVA analysis for EPA and DHA, and the FAs related to the synthesis of these LC-PUFAs (16:0, ALA, SDA, 16 MUFA, 18 MUFA), showed that the glucose assimilation explained (35%) the concentrations of these FAs in *Cryptomonas* sp., but the effect was rather

314 negative than positive (Fig. 2). It has been shown earlier that too high glucose concentration may
315 inhibit growth and lipid synthesis and that the optimal glucose content is species specific (Liang
316 et al. 2009; Wan et al. 2011). This effect was seen besides in the omega-3 in *Cryptomonas* sp.
317 also in the total FA content in *M. kalinae*, which had lowest total FAs in the highest glucose
318 treatment (5 mg L^{-1}).
319
320 In contrast to glucose addition, we expected that (Hypothesis 4) the leucine addition would not
321 affect the EPA and DHA content of algae, but could boost their growth and thus simultaneously
322 actually reduce the amount of stored FAs. However, the growth rates were not affected by
323 leucine, and reduction in the total FAs compared to control was found only in *M. kalinae* in
324 leucine 100 µg L^{-1} treatment. For our surprise, in PERMANOVA, leucine assimilation explained
325 48% of the variation in the content of 16:0, ALA, SDA, EPA, DHA 16 MUFA and 18 MUFA of
326 *Cryptomonas* sp., and - unlike expected - the effect of leucine was positive. Again, these results
327 show that biochemical synthesis in algae is species specific, and that generalizations cannot be
328 made.
329
330 We selected the glucose and leucine concentrations based on literature (Kamjunke & Tittel 2008;
331 Kamjunke et al. 2008), and they are in line with the dissolved organic carbon (DOC) contents of
332 natural lakes; the DOC content in the clearwater lakes in Finland vary between 7-9 mg C L^{-1}
333 (Ojala et al. 2011; Brek-Laitinen et al. 2012), whereas in humic lakes the DOC values can be
334 even higher (10 to 45 mg C L^{-1}; Taipale et al. 2008; Ojala et al. 2011). However, in nature the
335 DOC consists of both recalcitrant compounds from mainly terrestrial origin and labile
336 compounds released by algae and bacteria. Further, the labile compounds constitute of different
337 carbohydrates, organic acids, dissolved and free amino acids, ketones and aldehydes with
338 variable concentrations (Hellebust 1965; Norrman et al. 1995; Peltomaa & Ojala 2010;
339 Dąbrowska et al. 2014), which makes the detection of osmotropic assimilation as well as the
340 evaluation of its effects on e.g. FA synthesis challenging. In this study, we found some positive
341 and some negative effects of osmotrophic assimilation on FA synthesis, but the effects were still
342 minor in general, which agrees with the study of Galloway and Winder (2015), who reported that
343 growth conditions account for relatively low variation in algal FAs. However, in extreme
344 conditions, e.g. during enhanced run-off (either due to climate change or seasonality) leading to

higher carbon or amino acids in the water, the magnitude of the effects on LC-PUFA availability could be significant at food web level (from algae to fish; Jonasdottir 1994; Brett et al. 2006; Peltomaa et al. 2017; Taipale et al. 2018) assuming that the LC-PUFA producers of the algal community would consist of species capable on osmotrophic uptake of these compounds.

Conclusions

Our experiments show that osmotrophic nutrition can be found in different types of algae, but the assimilation is species specific and may differ between different organic compounds, as shown here with glucose and leucine. Furthermore, the effects of these two compounds on the algal growth and metabolism was found to be species specific: moderate glucose concentration (2 mg L^{-1}) enhanced the growth of *C. ozolinii*, whereas the growth of *M. kalinae* was enhanced by low leucine (20 µg L^{-1}). Additionally, high glucose content (5 mg L^{-1}) affected negatively on the total fatty acids of *M. kalinae* and the total omega-3 fatty acids of *Cryptomonas* sp. In general, glucose assimilation explained 35% (negative effect) and leucine assimilation 48% (positive effect) of the variation of EPA, DHA and the fatty acids related to their synthesis in *Cryptomonas* sp. but not in the other algae studied. The broad spectrum of compounds and the species-specific responses of algae makes the estimation of the importance of osmotrophy challenging in planktonic food webs and natural waters in general.

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Table 1(on next page)

Growth rates

Table 1. The maximal specific growth rates (μ; d^{-1}) of *Mallomonas kalinae*, *Cryptomonas* sp. and *C. ozolinii* in the long-term experiment. The growth rates were calculated for the exponential growth phase using equation 1. Replication n=3, *ANOVA $p < 0.01$, standard deviations are given in parenthesis.

Table 1. The maximal specific growth rates (μ; d^{-1}) of *Mallomonas kalinae*, *Cryptomonas* sp. and *C. ozolinii* in the long-term experiment. The growth rates were calculated for the exponential growth phase using equation 1. Replication n=3, *ANOVA $p < 0.01$, standard deviations are given in parenthesis.

Strain	Control	Glucose 0.5	Glucose 2	Glucose 5	Leucine 20	Leucine 100	Leucine 400
Mallomonas kalinae	0.20 (0.03)	0.20 (0.08)	0.23 (0.01)	0.20 (0.01)	0.47 (0.05)*	0.20 (0.04)	0.16 (0.06)
Cryptomonas sp.	0.21 (0.04)	0.23 (0.06)	0.36 (0.05)	0.26 (0.02)	0.23 (0.04)	0.25 (0.05)	0.28 (0.03)
Cryptomonas ozolinii	0.52 (0.01)	0.41 (0.01)	0.88 (0.07)*	0.34 (0.07)	0.34 (0.05)	0.65 (0.05)	0.57 (0.02)

Table 2(on next page)

Fatty acid results

Table 2. The concentrations (µg in mg dry weight) of 16:0 fatty acid (FA), alpha-linolenic acid (ALA; 18:3ω3), stearidonic acid (SDA; 18:4ω3), eicosapentaenoic acid (EPA; 20:5ω3), docosahexaenoic acid (DHA; 22:6ω3), total omega-3 (ω-3) FAs and total FAs in *M. kalinae, Cryptomonas* sp. and *C. ozolinii* in autotrophic controls and when grown osmotrophically with different glucose (i.e. 0.5 mg L^{-1}, 2 mg L^{-1}, 5 mg L^{-1}) and leucine (20 mg L^{-1},100 mg L^{-1},400 mg L^{-1}) concentrations in the long-term experiment. Replication n=3. Standard deviations are given in parenthesis. Different letters (a, b, c) denote significant differences (Tukey's HSD $p < 0.05$) between treatments, only statistically significant results are indicated.

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Strain	Fatty acid	Control	Glucose 0.5	Glucose 2	Glucose 5	Leucine 20	Leucine 100	Leucine 400
Mallomonas kalinae								
	16:0	8.2 (2.3)	4.1 (0.1)	7.9 (1.5)	5.0 (1.9)	8.1 (1.0)	4.0 (0.1)	6.8 (1.9)
	ALA	7.3 (0.0)^{a}	6.7 (4.1)^{ab}	8.3 (0.0)^{b}	4.8 (4.7)^{abc}	10.9 (1.1)^{b}	4.6 (0.3)^{c}	8.0 (1.1)^{ab}
	SDA	10.3 (1.6)^{a}	8.3 (6.3)^{a}	9.0 (1.4)^{a}	6.2 (5.4)^{ab}	12.9 (1.2)^{a}	5.6 (0.4)^{b}	10.6 (3.9)^{ab}
	EPA	0.3 (0.0)^{a}	0.2 (0.1)^{ab}	0.3 (0.0)^{a}	0.2 (0.1)^{ab}	0.3 (0.0)^{a}	0.1 (0.0)^{b}	0.3 (0.0)^{a}
	DHA	0.8 (0.0)	0.7 (0.5)	0.8 (0.1)	0.6 (0.3)	1.0 (0.1)	0.4 (0.0)	0.7 (0.2)
	Total FA	88.8 (5.8)^{a}	70.3 (8.7)^{ab}	74.1 (18.9)^{ab}	33.8 (14.8)^{bc}	63.8 (27.9)^{ab}	45.6 (0.3)^{b}	56.8 (22.3)^{ab}
Cryptomonas sp.								
	16:0	7.8 (1.0)^{ab}	7.8 (1.6)^{ab}	13.6 (0.4)^{b}	8.6 (0.7)^{ab}	10.4 (1.2)^{ab}	7.6 (2.0)^{ab}	10.2 (0.3)^{a}
	ALA	21.7 (3.7)^{ab}	16.8 (2.3)^{ab}	23.2 (0.3)^{a}	18.9 (3.2)^{ab}	20.1 (0.9)^{ab}	17.1 (1.2)^{ab}	18.0 (0.2)^{b}
	SDA	11.5 (2.2)	14.9 (1.3)	17.1 (2.6)	15.1 (3.8)	20.8 (2.2)	13.3 (4.0)	23.0 (1.2)
	EPA	13.1 (2.1)^{a}	11.5 (0.5)^{ab}	13.6 (3.3)^{ab}	12.3 (2.7)^{ab}	14.9 (2.5)^{ab}	11.1 (3.0)^{ab}	16.8 (0.4)^{b}
	DHA	1.4 (0.0)	2.0 (0.2)	2.1 (0.6)	1.8 (0.6)	2.8 (0.3)	1.7 (0.6)	3.4 (0.2)

	Total FA	60.7 (2.1)	52.8 (16.5)	73.6 (3.1)	70.5 (15.6)	67.7 (16.2)	55.9 (7.8)	68.8 (26.1)
Cryptomonas ozolinii								
	16:0	4.3 (0.0)	5.2 (1.5)	4.8 (1.5)	4.6 (0.2)	5.3 (1.0)	4.5 (0.2)	5.0 (0.1)
	ALA	8.1 (1.3)	10.1 (3.6)	9.5 (4.1)	9.0 (0.8)	10.9 (2.1)	9.2 (0.5)	8.5 (2.8)
	SDA	8.5 (0.2)	11.7 (4.2)	10.9 (6.1)	11.1 (0.8)	13.6 (1.7)	11.5 (1.2)	10.0 (3.7)
	EPA	7.0 (2.6)^{a}	8.9 (2.8)^{ab}	8.5 (4.5)^{ab}	9.1 (1.8)^{ab}	11.1 (0.1)^{b}	9.7 (1.6)^{ab}	8.2 (2.5)^{ab}
	DHA	1.6 (0.0)^{a}	2.2 (0.7)^{ab}	2.0 (1.2)^{ab}	2.4 (0.7)^{ab}	2.8 (0.0)^{b}	2.6 (0.5)^{ab}	2.0 (0.7)^{ab}
	Total FA	35.9 (7.1)	35.2 (0.3)	44.8 (18.0)	37.1 (4.6)	45.1 (16.9)	43.0 (9.6)	32.9 (2.8)

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Table 3(on next page)

PERMANOVA results

Table 3. Permutational multivariate analysis of variances (PERMANOVA) results for comparisons of the similarity of the concentrations of selected FAs (16:0, ALA, SDA, EPA, DHA, 16 MUFA, 18 MUFA) between the treatments in the long-term experiment. For the analysis, all glucose and all leucine treatments were pooled, thus replication n=9. SS sum of squares, MS, mean squares, P(perm) significance, P(MC) significance after Montecarlo correction. Statistically significant results are bolded.

Table 3. Permutational multivariate analysis of variances (PERMANOVA) results for comparisons of the similarity of the concentrations of selected FAs (16:0, ALA, SDA, EPA, DHA, 16 MUFA, 18 MUFA) between the treatments in the long-term experiment. For the analysis, all glucose and all leucine treatments were pooled, thus replication n=9. SS sum of squares, MS, mean squares, P(perm) significance, P(MC) significance after Montecarlo correction. Statistically significant results are bolded.

Strain	Treatment	df	SS	MS	Pseudo-F	P(perm)	P(MC)
Mallomonas kalinae	glucose	3	1697.1	565.71	1.4768	0.253	0.271
	leucine	3	1564.9	521.63	1.3617	0.331	0.339
Cryptomonas sp.	glucose	3	375.57	125.19	2.7389	**0.025**	**0.047**
	leucine	3	518.04	172.68	3.7779	**0.021**	**0.03**
Cryptomonas ozolinii	glucose	3	117.55	39.184	0.20727	0.918	0.898
	leucine	3	339.31	113.1	0.59828	0.702	0.654

Figure 1

Osmotrophic glucose and leucine assimilation

Figure 1. The osmotrophic glucose and leucine assimilation in the studied algal strains was detected with stable isotope labelling. The figures show the isotopic difference in ^{13}C (panel A) and ^{15}N (panel B) between the treatments in *Cryptomonas* sp., *Cryptomonas ozolinii*, *Chlamydomonas reinhardtii*, *Euglena gracilis*, *Mallomonas kalinae*, *Peridinium* sp. and *Selenastrum* sp. in the short-term experiment. The cultures were inoculated with ^{13}C-labeled glucose, ^{15}N-labeled leucine and mixture of these two. The total concentration of glucose in the experiment was 5 mg L^{-1} and leucine 400 mg L^{-1}. Replication n=3. The bars show standard errors, statistically significant difference between the non-labeled and labeled treatments are marked with star symbols (ANOVA, * $p < 0.5$, ** $p < 0.1$ and *** $p < 0.01$).

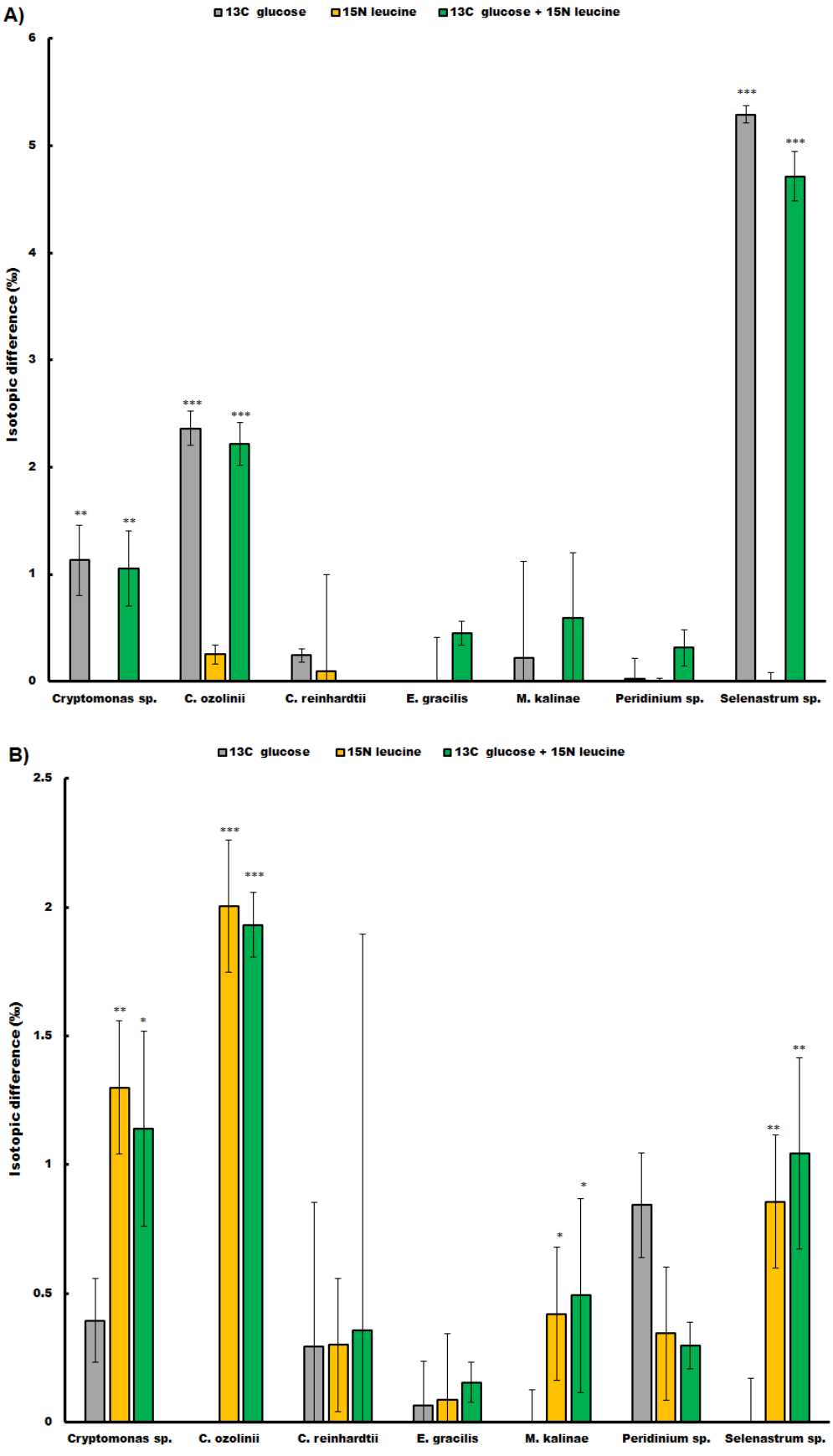
A)
13C glucose
15N leucine
13C glucose + 15N leucine
Isotopic difference (‰)
Cryptomonas sp.
C. ozolinii
C. reinhardtii
E. gracilis
M. kalinae
Peridinium sp.
Selenastrum sp.
B)
13C glucose
15N leucine
13C glucose + 15N leucine
Isotopic difference (‰)
Cryptomonas sp.
C. ozolinii
C. reinhardtii
E. gracilis
M. kalinae
Peridinium sp.
Selenastrum sp.

Figure 2

Fatty acid contents

Figure 2. The proportions of omega-3 FAs (alpha-linolenic acid, ALA; 18:3ω3, stearidonic acid, SDA; 18:4ω3, eicosapentaenoic acid, EPA; 20:5ω3, docosahexaenoic acid, DHA; 22:6ω3) on total omega-3 FAs in (A) *M. kalinae,* (B) *Cryptomonas* sp. and (C) *C. ozolinii* in autotrophic controls and when grown osmotrophically with different glucose (i.e. 0.5 mg L^{-1}, 2 mg L^{-1}, 5 mg L^{-1}) and leucine (20 mg L^{-1},100 mg L^{-1},400 mg L^{-1}) concentrations in the long-term experiment. Replication n=3.

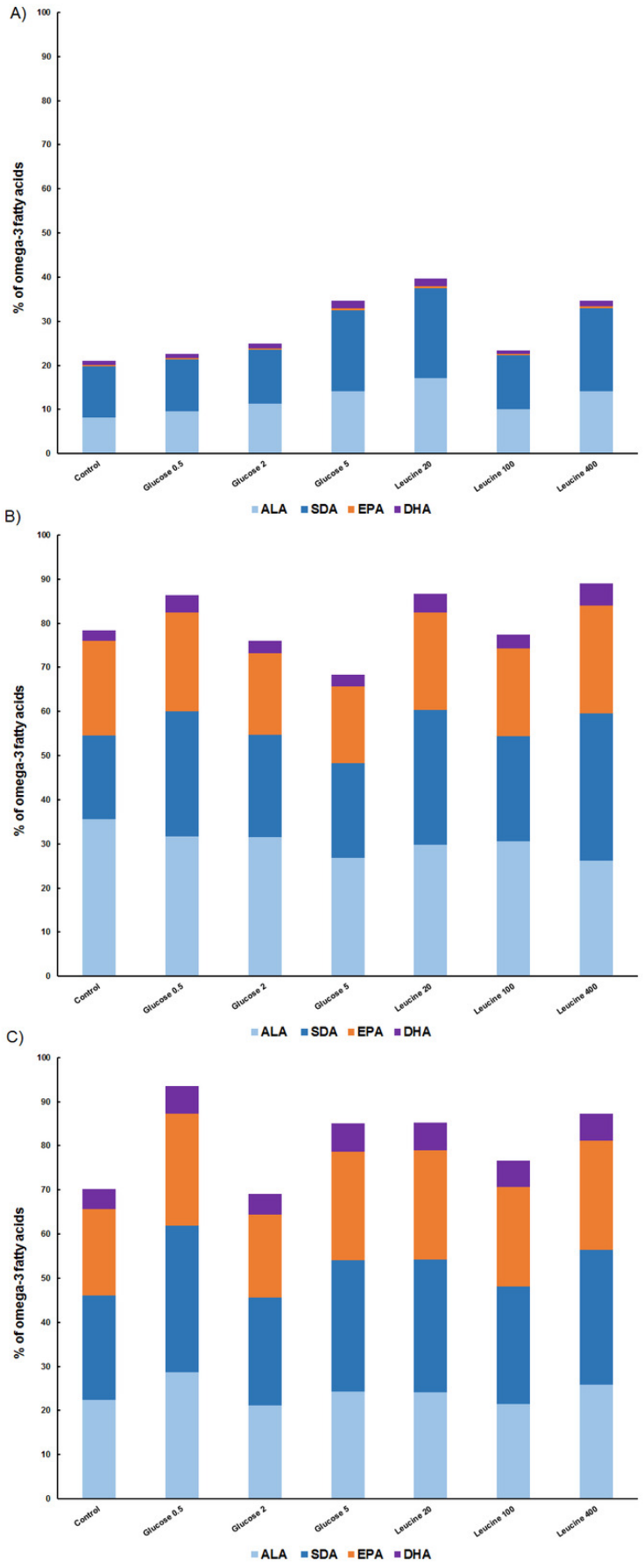
A)
% of omega-3 fatty acids
Control
Glucose 0.5
Glucose 2
Glucose 5
Leucine 20
Leucine 100
Leucine 400
ALA SDA EPA DHA
B)
% of omega-3 fatty acids
Control
Glucose 0.5
Glucose 2
Glucose 5
Leucine 20
Leucine 100
Leucine 400
ALA SDA EPA DHA
C)
% of omega-3 fatty acids
Control
Glucose 0.5
Glucose 2
Glucose 5
Leucine 20
Leucine 100
Leucine 400
ALA SDA EPA DHA

Figure 3

PCA plot

Figure 3. Principal component analysis (PCA) plot of the 16:0 fatty acid (FA), alpha-linolenic acid (ALA; 18:3ω3), stearidonic acid (SDA; 18:4ω3), eicosapentaenoic acid (EPA; 20:5ω3) and docosahexaenoic acid (DHA; 22:6ω3) of the long-term experiment showing that the studied three strains (*Mallomonas kalinae*, *Cryptomonas* sp. (CPCC 336) and *C. ozolinii*) differ from each other based on these FAs despite of the growth conditions (autotrophic, or osmotrophic with glucose or leucine; data shown in Table S2).