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Interacting effects of simulated eutrophication, temperature increase, and microplastic exposure on *Daphnia*

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Running head: *Daphnia* exposed to multiple stressors

ABSTRACT

The effects of multiple stressors are difficult to separate in field studies, and their interactions may be hard to predict if studied in isolation. We studied the effects of decreasing food quality (increase in cyanobacteria from 5 to 95% simulating eutrophication), temperature increase (by 3°C), and microplastic exposure (1% of the diet) on survival, size, reproduction, and fatty acid composition of the model freshwater cladoceran *Daphnia magna*. We found that food quality was the major driver of *Daphnia* responses. When the amount of cyanobacteria increased from 5 to 95% of the diet, there was a drastic decrease in *Daphnia* survival (from 81 ± 15% to 24 ± 21%), juvenile size (from 1.8 ± 0.2 mm to 1.0 ± 0.1 mm), adult size (from 2.7 ± 0.1 mm to 1.1 ± 0.1 mm), and reproduction (from 13 ± 5 neonates per surviving adult to 0), but the decrease was not always linear. This was most likely due to lower availability of lipids, eicosapentaenoic acid (EPA), and sterols from the diet. Microplastic exposure did not affect *Daphnia* survival, size, or reproduction. Food quality had an interactive effect with temperature on fatty acid content of *Daphnia*. Total fatty acid content of *Daphnia* was almost 2-fold higher at 20°C than at 23°C when fed 50% cyanobacteria. This may have implications for higher trophic level consumers, such as fish, that depend on zooplankton for energy and essential lipids. Our findings suggest that as proportions of cyanobacteria increase, in tandem with water temperatures due to climate change, fish may encounter fewer and smaller *Daphnia* with lower lipid and EPA content.

Key words: cyanobacteria, fatty acids, climate change, food quality *Daphnia magna*

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Abbreviations:

EPA = eicosapentaenoic acid

PUFA = polyunsaturated fatty acid

MP = microplastic

cyano = cyanobacteria

PET = polyethylene terephthalate

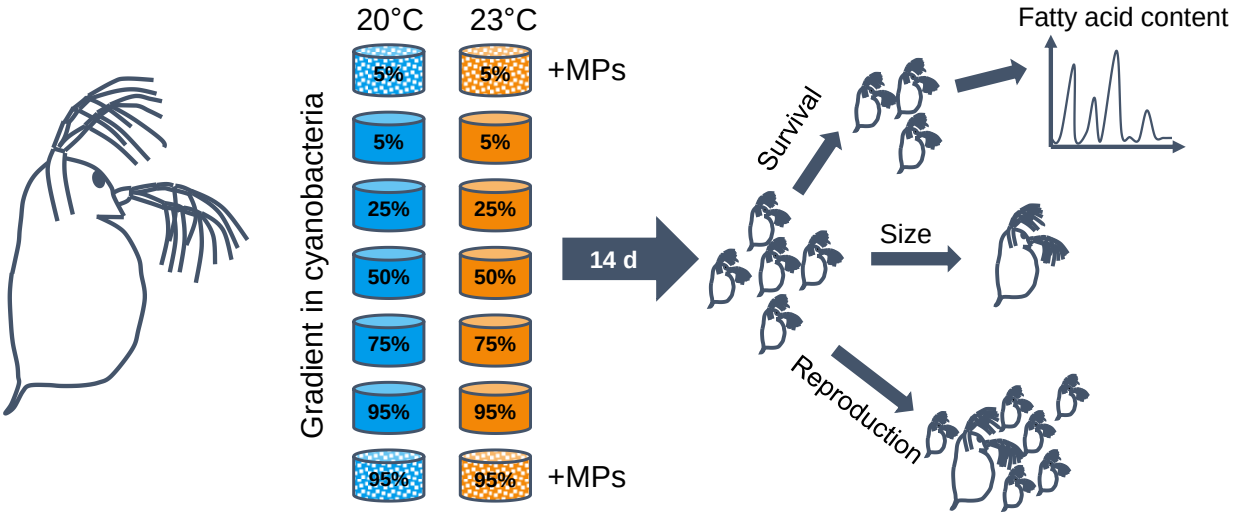
PS = polystyrene

ABS = acrylonitrile butadiene styrene

PERMANOVA = Permutational multivariate analysis of variance

DistLM = distance-based linear modeling

GRAPHICAL ABSTRACT



Daphnia magna were grown along a gradient in cyanobacteria (5-95%, mimicking eutrophication) in ambient and +3°C temperature with and without exposure to a mix of secondary microplastics (MPs). Proportion of cyanobacteria in diet was the major driver of *Daphnia* fitness, and had minor interactions with temperature, while we found no effects of MPs.

1. INTRODUCTION

Eutrophication, climate change induced temperature increase, and microplastic contamination are among the biggest challenges that aquatic organisms face today. Excessive nutrient loading results in high phytoplankton biomass consisting mainly of cyanobacteria, which in addition to potentially being toxic, are poor quality resources for zooplankton (Lampert 1987; Paerl and Paul 2012; Huisman et al. 2018). Surface water temperatures are increasing (O'Reilly et al. 2015), which has a fundamental effect on small- and large-scale processes in aquatic ecosystems including biochemical reactions inside the cells, life-history traits of organisms, and water movement and stratification patterns (Adrian et al. 2009). During recent years, microplastics (often defined as <5 mm) have been found in all aquatic systems, ranging from freshwater lakes and streams to the ocean abyss, and their effects on organisms are under intensive investigation (Li et al. 2018; de Sa et al. 2018). These stressors act in unison, and studying them separately may not reveal all the potential impacts they may have on organisms that are facing multiple stressors in the natural environment.

Food quality may be more important in explaining variation in zooplankton fitness than food quantity (Müller-Navarra et al. 2000). Fatty acids and sterols in diet influence *Daphnia* growth, reproduction, and lipid composition (Becker & Boersma 2005; Brett et al. 2006; Martin-Creuzburg et al. 2009; Peltomaa et al. 2017). Lipids are key components of all cells – their composition influences the fluidity, permeability, and protein action of biological membranes, and additionally, lipids act as energy storage material and function as precursors for hormone-like signaling molecules such as eicosanoids (Arts and Kohler 2009). The production of polyunsaturated fatty acids (PUFAs, with two or more double bonds in carbon chain) is mostly restricted to plants, and animals need to acquire PUFAs from their diet for optimal growth and

reproduction (Arts and Kohler 2009). Phytoplankton produce phytosterols, which consumers transform to cholesterol (Martin-Creuzburg and von Elert 2009). Cholesterol together with fatty acids regulate cell membrane structure and function (Stillwell & Wassal 2003). Sterols are also needed in production of vitamin D precursors and ecdysteroids, hormones, which are involved in for example molting of arthropods (Martin-Creuzburg and von Elert 2009). Cyanobacteria are a poor quality resource for zooplankton due to their tendency to form large colonies and filaments, production of harmful secondary metabolites (Paerl and Paul 2012), and lack of sterols and long-chain PUFAs (≥ C20) (Martin-Creuzburg et al. 2008, 2009). Lake phosphorus concentrations are negatively correlated with the availability of essential PUFAs in the seston, resulting in reduced growth rates and reproduction of zooplankton in eutrophic lakes (Müller-Navarra et al. 2004). Furthermore, increased abundance of cyanobacteria restricts energy and PUFA transfer in the food web and ultimately results in fish being lower quality resources for human nutrition (Taipale et al. 2016b). In contrast to cyanobacteria, diatoms and cryptophytes have abundant long-chain PUFAs and sterols to support high growth rates in zooplankton (Galloway and Winder 2015; Taipale et al. 2016a; Peltomaa et al. 2017). Green algae lack long-chain PUFAs, but contain sterols, and their ingestion generally results in intermediate growth and reproduction of *Daphnia* (Hiltunen et al. in 2017; Peltomaa et al. 2017).

Nutritional requirements of animals may change in response to temperature shifts. Climate scenarios predict that by 2100 air temperatures in the temperate zone will likely rise 0.3 to 4.8°C compared to the 1986-2005 levels due to anthropogenic climate change (IPCC 2014). More severe increases in temperature are expected in the polar regions. Additionally, extreme weather events, such as heat waves and droughts are expected to become more common, resulting in organisms facing episodic thermal stress. Faster metabolic rates of ectothermic animals in higher

temperatures impose higher metabolic costs that can only be met if there is enough food available (Gillooly et al. 2001). Furthermore, *Daphnia* require more PUFAs in colder temperatures, and more sterols in higher temperatures, presumably to maintain membrane stability (Sperfeld and Wacker 2009, 2012). Thus, increasing temperature may either enhance organism fitness by increasing growth rates, or act as an additional stress factor if the animal is already in the upper thermal tolerance limit or in poor nutritional state. Consumers may increase feeding rates at high temperatures, exposing them to higher levels of contaminants present in water (Folt et al. 1999). The interactions of nutrition, temperature and contaminants have been very rarely studied, and to our knowledge, only in the context of food quantity, not quality (Folt et al. 1999; Heugens et al. 2006).

Plastic contamination is ubiquitous in all aquatic environments, but the occurrence of microplastics in freshwater systems and their effects on biota are less extensively studied compared to marine systems (Li et al. 2018; de Sa et al. 2018). The limited number of studies done so far have found that the abundance of microplastics in freshwater habitats reaches up to 187 particles L^{-1} (reviewed by Li et al. 2018). Lenz et al. (2016) highlighted that exposure concentrations in many laboratory studies have been several magnitudes higher (up to 10^{12} particles L^{-1}) than those actually measured in the environment, which restricts the applicability of their results in the real world. For example, high levels of nanoplastics or secondary microplastics in *Daphnia* diet caused elevated mortality and decreased reproduction (Besseling et al. 2014; Ogonowski et al. 2016). Imhof et al (2017) found no effects of secondary microplastics on *Daphnia* survival when using a more realistic exposure concentration, although still higher than found in the field (1% of food particles or 290,000 particles L^{-1}), but gene expression data indicated small increases in stress responses together with inconsistent

differences in morphological traits of offspring. Furthermore, Ogonowski et al. (2016) found irregularly-shaped secondary microplastics with broad size distribution to be more harmful to *Daphnia* than primary microplastics, suggesting that results from exposure studies using pristine microplastics (mainly polystyrene beads) might underestimate the impacts in the environment where secondary microplastics are more common. Moreover, microplastic particles in nature become rapidly coated with biofilm (Rummel et al. 2017), which might further affect their uptake or effects on organisms. Very few studies have so far investigated the effects of microplastics together with other environmental stressors (but see Ferreira et al. 2015; Aljaibachi and Callaghan 2018; Jaikumar et al. 2018).

We conducted a laboratory study to investigate how *Daphnia magna*, a model freshwater zooplankton species, reacts to multiple stressors of eutrophication (increase in cyanobacteria), climate change (increase in temperature), and microplastic exposure. To our knowledge, the interactions of food quality, temperature, and contaminants have not been previously studied. Although the study was done in a laboratory setting, the aim was to keep the levels environmentally relevant to better predict outcomes in the field. We used a gradient in the proportion of cyanobacteria in diet from 5 to 95% to mimic the transition in food quality that takes place from oligotrophic to hyper-eutrophic systems. The chosen temperature increase was 3°C, corresponding to Representative Concentration Pathway 6.0 (RCP 6.0) projections of IPCC for northern Europe by the end of the century (IPCC 2014). Temperature in lake surface waters is increasing at 0.34 °C decade^{-1} (O'Reilly et al. 2015), potentially leading to 3°C increase in a 100-year time scale. Microplastic exposure was kept at 1% of total food concentration, which at 0.03 mg C L^{-1} (or 307,000 particles L^{-1}) is higher than recorded for freshwater systems, nevertheless representing only a small fraction of the diet. To better mimic natural conditions, we

used a mix of secondary microplastics, which was inoculated with microbes from lake water to promote biofilm formation. Our hypotheses were: 1) high food quality (low proportion of cyanobacteria) will lead to increased survival, size, and reproduction of *Daphnia*, 2) temperature increase will improve *Daphnia* performance when cyanobacteria forms a low proportion of the diet, 3) temperature increase and microplastic exposure will decrease *Daphnia* fitness when cyanobacteria forms a high proportion of the diet.

2. MATERIALS AND METHODS

2.1 Materials

We conducted a laboratory experiment to investigate the interactions between food quality (in reference to eutrophication), temperature increase, and microplastic exposure on zooplankton. *Daphnia magna* (hereafter *Daphnia*) was used as the model organism, and the experiment was started with neonates, which had hatched from ephippia (Daphtox kit F magna by MicroBioTests Inc.). The aim was to use neonates <24 h old, but neonates hatched earlier than anticipated, and were 24 to 40 h old when the experiment started. The filter-feeding *Daphnia* are keystone species that can control phytoplankton and bacterial abundance, while themselves being preferred food for fish. *Daphnia magna* is widespread in temperate and boreal freshwater environments and exhibits a temperature optimum of ca. 23-29°C depending on clone origin (Mitchell and Lampert 2000), and thus may benefit from future increases in surface water temperatures in the temperate zone.

We used three algae in the experiment: a non-toxic strain of the cyanobacteria *Microcystis aeruginosa* (CPCC 633), the green algae *Acutodesmus* sp. (University of Basel) and the diatom

Nitzschia sp. (W6 by Czech Republic Academy of Science). Parallel measurements of turbidity (in NTU, WTW TURB 430 IR), biomass (in mg DW L^{-1}), and carbon content (C% of DW) were used to create turbidity to carbon concentration equations to calculate the amounts of each algae in the treatments. Biomass was measured by filtering a known volume of algal culture on preweighted GF/A filters, drying filters overnight at 60°C, and weighing them again. The carbon content of algae was analyzed from centrifuged and freeze-dried material with a Carlo-Erba Flash 1112 series Element Analyzer connected to a Thermo Finnigan Delta Plus Advantage IRMS at the University of Jyväskylä, Finland. Dried and ground spirulina was used as a laboratory working standard. The resulting equations were: carbon concentration (mg C L^{-1}) = 1.55*turbidity – 3.74 for *Acutodesmus* (R^2 = 0.989), carbon concentration (mg C L^{-1}) = 1.49*turbidity – 2.36 for *Microcystis* (R^2 = 0.961), and carbon concentration (mg C L^{-1}) = 0.409*turbidity – 2.83 for *Nitzschia* (R^2 = 0.983).

For the microplastic exposure treatments, we produced secondary microplastics from a soda bottle (polyethylene terephthalate, PET), polystyrene (PS) tray, and toy brick (acrylonitrile butadiene styrene, ABS). The material was ground with a kitchen grinder, dissolved in MQ water and homogenized with an Ultra Turrax for 5 min in crushed ice. The stock suspensions were sieved with a 50 µm screen to remove large particles that *Daphnia* are not able to ingest. The particle numbers and size-distribution of microplastics were analyzed with a particle counter (CASY Cell Counter and Analyzer, Omni Life Science) using a 60 µm capillary (for size-range 1.2–40 µm). The protocol resulted in a vast majority of the particles being <10 µm (see Supplemental figure 1 for size-distributions). The mean particle diameter was 3.2 – 3.7 µm, but peak particle diameter was 1.6 µm, indicating that some of the particles fell below the detection limit of the particle counter. Thus, the actual particle numbers during the exposure experiment

were likely somewhat higher than calculated. The suspensions were left to stand for 48 h and then washed with repeated centrifugation and resuspension in clean MQ water to remove any additives potentially leaching from the material. The microplastics were then suspended in 50 mL MWC media and inoculated with 1mL of water from Lake Päijänne to introduce a natural microbial community. The stock suspensions were incubated for 7 days in room temperature, and then stored in a refrigerator during the experiment. We assumed a density of 1.04 g cm^{-3} for PS, 1.38 g cm^{-3} for PET, and 1.07 g cm^{-3} for ABS, and analyzed their carbon content with the same method as the algae to calculate the amounts added to the feeding mix, which was used to prepare the *Daphnia* growth media during the experiment. The particle numbers (particles mL^{-1}) and concentrations (mg C mL^{-1}) of microplastics in the stock solutions are presented in Table 1.

2.2 Experimental setup

The treatments consisted of a 5-point cyanobacteria gradient (from 5 to 95% of the diet), mimicking the transition in food quality from oligotrophic (low cyanobacteria) to eutrophic (high cyanobacteria) systems, with equal shares of the green algae and diatoms making up the remaining proportion of the diet (Table 2) conducted in two temperatures (20°C and 23°C). Additionally, the lowest and the highest cyanobacteria treatment (5% and 95% of diet) in both temperatures were run with microplastic exposure (1% of diet), bringing the total number of treatments to 14 (Figure 1). Each replicate consisted of 10 randomly selected *Daphnia* neonates in a 400 ml beaker with 200ml of pre-aerated artificial lake water (OECD 2004), and each treatment had 4 replicates. *Daphnia* were grown in batches, and not as single individuals, to gain enough material for fatty acid analysis.

The algae were grown in either 20°C or 23°C, corresponding to the temperature treatments in the *Daphnia* experiment, as suggested by von Elert and Fink (2018). The algae were cultured in MWC medium (Guillard and Lorenzen 1972) with added vitamins in a 16:8h light:dark cycle with light intensity of 2 350 -2 470 lux. Algae were grown in 2-3L Erlenmeyer flasks and two flasks were starters for each algae and temperature. When algae were fed to *Daphnia*, bottles were refilled with fresh MWC medium to keep the algae growing exponentially. We switched between the two bottles of *Microcystis* and *Nitzschia* every two feeding days, because they became too diluted, but used the same bottles for *Acutodesmus* throughout the experiment. Phytoplankton were sampled on days 2, 6, 10, and 14 by centrifugation, and stored in freezer (-80°C) for subsequent fatty acid analysis. The experiment was conducted in a temperature controlled room (water temperature 19.8 ± 0.5°C) with the *Daphnia* and algae for 23°C treatments grown in water baths (measured temperatures during the experiment 23.1 ± 0.2°C and 23.0 ± 0.1°C, respectively). Only the bottom of the phytoplankton bottles was submersed in the water bath to avoid differences in light intensity. The experiment lasted 14 days. Every other day the *Daphnia* were counted and transferred to fresh food suspensions. Food was offered at concentrations of 3 mg C L^{-1}.We harvested and measured 1-2 *Daphnia* per replicate on day 6 to determine the juvenile size. The body length of *Daphnia* was measured from the tip of the head to the base of the tail spine. The produced offspring were counted to measure reproductive capacity (neonates per surviving adult). At the end of the experiment, *Daphnia* were harvested and three per replicate (if that many survived) were measured to determine their final length, and all individuals were frozen (-80°C) for fatty acid analysis about a week later.

2.3 Fatty acid analysis

Fatty acids from *Daphnia* were analyzed as two replicates per treatment, except for treatments with 75% cyanobacteria where we could only analyze one replicate (per temperature) due to low sample material. For treatments with 95% cyanobacteria we had to pool all replicates in both temperatures with and without microplastics to get enough material for one fatty acid analysis. One replicate of the 25% cyanobacteria in 23°C treatment was lost during analysis, and also during reanalysis from excess material. This resulted in total $n = 18$ for *Daphnia* fatty acid analysis. We analyzed the three phytoplankton taxa grown in both temperatures on days 2, 6, 10, and 14, resulting in total $n = 24$ for phytoplankton fatty acids. The *Daphnia* and phytoplankton were freeze-dried, weighed, and subjected to fatty acid analysis. Lipids were extracted twice with chloroform:methanol (2:1, v/v), and non-lipid components were removed by washing with MQ-water following Folch et al. (1957). The solvent was evaporated under an N_2 stream, and n-hexane and 1% H_2SO_4 in methanol was added. The transmethylation of the fatty acids was facilitated by heating the samples in a water bath at 90°C for 90 min. The produced fatty acid methyl esters were extracted twice with n-hexane, concentrated with N_2, transferred to small vials, and then run with a gas chromatograph-mass spectrometer (GCMS-QP2010 ultra, Shimadzu). The column was ZB-FAME (Phenomenex) and helium was used as the carrier gas with velocity 36 cm s^{-1}. The sample was injected splitless and the inlet temperature was 270°C. The initial oven temperature of 50°C was held for 1 min, then raised 10°C min^{-1} to 130°C, 7°C min^{-1} to 180°C, 2°C min^{-1} to 200°C and held for 3 min. For quantification, we used 23:0 as the internal standard and the GC-MS response was calibrated with a concentration series of a standard mix (566C, Nu chek prep.).

2.4 Statistical tests

We used a General Linear Model to test whether the proportion of cyanobacteria in diet (as a covariate) or rearing temperature (factor) or their interaction affected the size and fatty acid composition of *Daphnia*. Juvenile length was log(x+1) transformed to meet the assumptions on equality of variance across groups. Regression models (linear and polynomial) were used to explore how well food quality (proportion of cyanobacteria in diet) explained the variance in length, total fatty acids content, and omega-3 to omega-6 ratio of *Daphnia*. For the response variables that were in counts (survival, neonate production) we used a Generalized Linear Model with Poisson distribution and log link to test for the effects of proportion of cyanobacteria in diet (covariate) and rearing temperature (factor). The natural logarithm of the number of animals in the beginning (adjusted for animals removed on day 6) was used as the offset value for survival in the model, while the natural logarithm of animals alive at the end of the experiment was used as the offset for reproduction. The non-parametric Krukal-Wallis H-test with Dunn-Bonferroni post hoc pairwise comparisons was used for testing differences between treatments with and without the microplastic exposure due to violation of assumptions for 1-ANOVA. Differences in the percent composition of algal fatty acids among taxa and temperatures were examined using Permutational multivariate analysis of variance (PERMANOVA), which can be used for multivariate data sets from ANOVA-type experimental designs (Anderson et al. 2008). PERMANOVA was run with type III sum of squares and unrestricted permutation of raw data We used distance-based linear modeling (DistLM) to see if proportion of cyanobacteria in diet explained the variation in fatty acid percent composition of *Daphnia*. DistLM models the relationship between predictor variable(s) and a multivariate response data set in a similar way that regression is used to model the relationship between the predictor variable(s) and a single response variable (Anderson et al. 2008). The full fatty acid dataset (in proportions) is included as

Supplemental Table S1. The statistical analysis was run with IBM SPSS 24 or Primer 6 and PERMANOVA+ add-on.

3. RESULTS

3.1 Survival

Daphnia survival was on average 81 ± 15% (mean ± SD) when fed 5% cyanobacteria (both temperatures and microplastic exposures pooled). Survival of *Daphnia* decreased when cyanobacteria contributed 50% or more to their diet (Generalized linear model, $p < 0.001$, Figure 2A, Table 3) and was 24 ± 21% when fed 95% cyanobacteria. The main test indicated differences in *Daphnia* survival when fed 95% cyanobacteria with temperature (Kruskal-Wallis, $\chi^2 = 10.81$, $p = 0.012$, Figure 2B), but the pair-wise differences were not significant (Bonferroni-corrected Dunn test, $p > 0.1$). *Daphnia* survival was not affected by temperature or microplastics when fed 5% cyanobacteria (Kruskal-Wallis, $\chi^2 = 3.80$, $p = 0.284$).

3.2 Juvenile and adult size

Daphnia juvenile size was highly influenced by the amount of cyanobacteria in diet (general linear model, $p < 0.001$, Table 4) but not by temperature ($p = 0.173$), with high proportions of cyanobacteria leading to smaller *Daphnia* (linear regression, $R^2 = 0.817$, $F = 169.417$, $p < 0.001$, Figure 3A). *Daphnia* juvenile size decreased from 1.8 ± 0.2 mm to 1.0 ± 0.1 mm when proportion of cyanobacteria in diet increased from 5 to 95%, respectively. Microplastic exposure or temperature had no effect on *Daphnia* juvenile size when fed 5% or 95% cyanobacteria (Kruskal Wallis, $\chi^2 = 1.217$, $p = 0.749$, and $H = 2.377$, $p = 0.498$, respectively) (Figure 3B). Similarly *Daphnia* final length (measured after 14 days) was highly influenced

by the amount of cyanobacteria in the diet ($p < 0.001$, Table 4) but not by temperature, and there was no interaction between diet and temperature (Figure 4A). *Daphnia* adult size decreased from 2.7 ± 0.1 mm to 1.1 ± 0.1 mm when proportion of cyanobacteria in diet increased from 5 to 95%, respectively. Microplastic exposure or temperature had no effect on *Daphnia* final length when fed 5% or 95% cyanobacteria ($\chi^2 = 1.150$, $p = 0.765$, and $\chi^2 = 4.303$, $p = 0.231$, respectively) (Figure 4B). After being fed 5% cyanobacteria (with temperatures and microplastic exposures pooled) for 14 days *Daphnia* were on average 2.7 ± 0.1 mm long, and when fed 95% cyanobacteria only 1.1 ± 0.1 mm long.

3.3 Reproduction

The first neonates were observed on day 10 of the experiment in treatments receiving 5% cyanobacteria, on day 12 in 25% cyanobacteria treatments, and on day 14 in 50% cyanobacteria treatments (except in one replicate on day 12), while *Daphnia* receiving 75% or 95% cyanobacteria did not produce offspring during the experiment. Reproductive output of *Daphnia* decreased when the proportion of cyanobacteria in their diet increased (Generalized linear model, $p < 0.001$, Table 3, Figure 5A), while temperature had no effect ($p = 0.662$). Microplastic exposure or temperature did not affect the reproductive output when fed 5% cyanobacteria (Kruskal-Wallis, $\chi^2 = 4.260$, $p = 0.235$, Figure 5B). *Daphnia* produced on average 13 ± 5 neonates per surviving adult when fed 5% cyanobacteria (both temperatures and MP exposures pooled), 7 ± 2 when fed 25% cyanobacteria, and 1 ± 1 when fed 50% cyanobacteria.

3.4 Fatty acid content and composition of *Daphnia* and phytoplankton

The total fatty acid content (µg mg DW^{-1}) was lower in *Daphnia* receiving high proportion of cyanobacteria in diet, but there were differences between the temperature treatments (general linear model, $p = 0.002$, Table 4) (Figure 6A). Total fatty acid content decreased linearly with

increasing cyanobacteria in *Daphnia* reared at 23°C (linear regression, $F = 40.977$, $R^2 = 0.872$, $p = 0.001$), while *Daphnia* sustained a high fatty acid content when fed up to 50% cyanobacteria at 20°C, resulting in a quadratic relationship (2nd order polynomial regression, $F = 10.588$, $R^2 = 0.779$, $p = 0.011$). The total fatty acid content when fed 50% cyanobacteria was almost two-fold higher in *Daphnia* reared at 20°C than at 23°C. Furthermore, the fatty acid percent composition of *Daphnia* was influenced by the amount of cyanobacteria in their diet (DistLM, F = 26.12, $p < 0.001$, $R^2 = 0.620$). With increasing amount of cyanobacteria, *Daphnia* had more saturated fatty acids and 18:3ω6 (indicative of *Microcystis*), and less C16PUFAs and 18:3ω3 indicative of *Nitzschia* and *Acutodesmus* (Supplemental Table 1, Figure 7). The ω-3:ω-6 ratio in *Daphnia* was lower when reared in the higher temperature and decreased with increasing cyanobacteria in the diet (Figure 6B, Table 4). We found no differences in fatty acid composition or total content in *Daphnia* with or without microplastic exposure at 5% cyanobacteria. However, we needed to pool the exposed and non-exposed *Daphnia* that had been fed 95% cyanobacteria for fatty acids due to very low amounts of sample material.

Total fatty acid content differed among the phytoplankton taxa (general linear model, $F = 14.701$, $p < 0.001$), but not between temperatures ($F = 0.001$, $p = 0.981$), and there were no interactions between the factors ($F = 1.210$, $p = 0.321$). *Microcystis* had a lower total fatty acid content (51 ± 12 µg mg DW^{-1}) than *Acutodesmus* or *Nitzschia* (102 ± 24 and 86 ± 20 µg mg DW^{-1}, respectively) (Bonferroni-corrected pair-wise comparisons, $p < 0.01$). Fatty acid percent composition differed greatly between the three phytoplankton taxa with taxonomic identity explaining 95% of the variation in the fatty acid data (PERMANOVA, $F_{2,18} = 433.72$, $p < 0.001$).

There also were differences in algal fatty acid composition between the temperatures ($F_{1,18} = 170.70$, $p = 0.006$), and an interaction between taxa and temperature ($F_{2,18} = 351.78$, $p =$

0.001). The fatty acid composition of *Microcystis* differed between the temperatures (t = 6.173, p(MC) < 0.001) but there was no difference in *Acutodesmus* (t = 1.239, p(MC) = 0.233) or *Nitzschia* (t = 1.823, p(MC) = 0.117). *Microcystis* was rich in the saturated fatty acid (SAFA) 16:0, which made up 46.8 ± 0.5% of its fatty acids (Figure 7). *Microcystis* lacked long-chain PUFAs, except for a small quantity of 20:3ω6 (0.1 ± 0.1%), but had abundant ω-3 C18PUFAs and ω-6 C18PUFAs. The share of ω-6 C18PUFAs increased from 15.5 ± 0.7% to 24.3 ± 1.2% and ω-3 C18PUFAs decreased from 26.9 ± 1.4% to 17.4 ± 0.6% with the increased temperature. *Acutodesmus* also lacked long-chain PUFAs, but was very rich in 18:3ω3 (44.5 ± 1.2%) and 16:4ω3 (17.3 ± 1.0%). *Nitzschia* had a high proportion of the monounsaturated fatty acid (MUFA) 16:1ω7 (27.9 ± 6.6%) and C16 and C18 ω-4 PUFA (15.1 ± 4.2%). In contrast to *Microcystis* and *Acutodesmus*, *Nitzschia* lacked ω-3 C18PUFAs almost entirely, but was very rich in the long-chain PUFA eicosapentaenoic acid (EPA, 20:5ω3; 23.9 ± 5.9%).

We found that the *Nitzschia* bottle used for feeding the *Daphnia* grown at 23°C on day 10 was contaminated with *Microcystis* cells. The fatty acid data shows slightly elevated levels of 18:3ω6 (1.8%) in that sample compared to other *Nitzschia* samples (≤ 0.5%), but fatty acids characteristic of *Nitzschia* still formed a majority of fatty acids in this sample, indicating only minor contamination. Nevertheless, the *Daphnia* in 23°C treatments received slightly higher proportions of cyanobacteria than the *Daphnia* in 20°C on this single feeding day. This bottle was previously used on day 4 with no signs of contamination, and was not used after the contamination was discovered.

4. DISCUSSION

We conducted a laboratory experiment to investigate the interacting effects of eutrophication, climate change, and microplastic exposure on the model freshwater cladoceran *Daphnia magna*. *Daphnia* were reared on a gradient of food quality (proportion of cyanobacteria) in two temperatures with and without microplastic exposure, and we found that food quality was the most important factor determining the fitness of *Daphnia*. Cyanobacteria generally contain neither long-chain PUFAs nor sterols (Galloway and Winder 2015; Taipale et al. 2016a), although we found very small amounts of 20:3ω6 in our *Microcystis aeruginosa* strain. The lack of sterols is the predominant reason for low somatic growth in *Daphnia* fed cyanobacteria, while the lack of PUFAs has a strong influence on reproduction and population growth (Martin-Creuzburg et al. 2008). The green algae *Acutodesmus* sp. also lacked EPA, in contrast to the diatom *Nitzschia*, which was rich in EPA. Both green algae and diatoms contain sterols (Taipale et al. 2016a; Peltomaa et al. 2017). Thus, in our experiment, *Daphnia* receiving increasing amounts of cyanobacteria had lower amounts of EPA and sterols available in their diet, which likely explains the drastic effects on survival, juvenile and adult size, reproduction, and fatty acid content.

We found that the response of cyanobacteria on *Daphnia* reproduction was non-linear, while the size of *Daphnia* decreased linearly when exposed to increasing cyanobacterial abundances. Reproduction was high when cyanobacteria formed 5% of the diet and decreased rapidly after that. Thus, from the parameters we measured, reproduction exhibited the strongest response to increasing cyanobacterial abundances. In contrast, Martin-Creuzburg et al. (2005) found the responses of growth and reproduction of *D. magna* to be similar, with decrease in both when cyanobacteria formed ≥50% of the diet, while 20% of cyanobacteria did not cause and effect.

Temperature is a crucial factor affecting the physiological processes of ectothermic organisms in the aquatic environment. *D. magna* can tolerate a wide range of temperatures

with highest growth rates observed in >25°C for many clones (Mitchell and Lampert 2000). *Daphnia* also experience seasonal variation in temperature, which may drive reproductive output (George et al. 1990), however, even small deviations (<2°C) in temperature during critical periods in the seasonal cycle may have drastic effects on *Daphnia* populations (Wagner & Benndorf 2007). In the present study, temperature did not affect size or reproduction of *Daphnia*, but it had an interactive effect with food quality on *Daphnia* fatty acid content. Previous research has highlighted that *Daphnia* fitness is strongly affected by sterols and PUFAs (especially EPA) in their diet (Müller-Navarra et al. 2000; Becker & Boersma 2005; Martin-Creuzburg et al. 2009; Peltomaa et al. 2017). EPA functions as an important component of cell membranes and is also linked to eicosanoid production in *Daphnia* (Farkas 1979; Schlotz et al. 2012). Maintaining membrane properties is vital when acclimating to temperature (Guschina and Harwood 2006), and *Daphnia* has been found to increase the unsaturation of membrane lipids in colder temperatures (Farkas 1979). This results in a higher dietary demand of PUFA in lower temperatures, and *Daphnia* growth is more strongly limited by EPA in 10 to 15°C than 20 to 25°C (Masclaux et al. 2009; Sperfeld and Wacker 2011, 2012; Martin-Creuzburg et al. 2012; von Elert and Fink 2018), which is the range where our experiment was conducted. However, at higher temperatures (25°C) cholesterol seems to be important for *Daphnia* growth and reproduction (Sperfeld and Wacker 2009; Martin-Creuzburg et al. 2012). In contrast to our hypothesis, we did not observe an increase in growth of *Daphnia* at the higher temperature, even when the food quality was high and microplastics absent. It is possible that the temperatures we chose were so close to each other, and also close to the optimum value for our *Daphnia magna* strain that it did not cause large differences.

Cyanobacteria blooms often coincide with high temperatures, and it has been predicted that climate change will result in an increase in the prevalence and intensity of cyanobacterial

blooms (Paerl and Paul 2012; Huisman et al. 2018). Thus, consumers in future lakes may face decreased food quality with higher temperatures. A previous study found that the effect of temperature and poor food quality together on *Daphnia pulex* were greater than their effects separately (Przytulska et al. 2015). In the present study, *Daphnia* survival seemed to actually be higher with the higher temperature when the food quality was low, in contrast to our hypothesis, but this effect was not statistically significant. However, *Daphnia* reared at 23°C with 95% cyanobacteria were just as small as the *Daphnia* at 20°C, and did not produce any offspring, indicating that higher temperature would not prevent the population from collapsing under hyper-eutrophic conditions. We also found that the lipid accumulation patterns in *Daphnia* were driven by temperature when exposed to a gradient in food quality. When the proportion of cyanobacteria in the diet was low (5%), *Daphnia* total fatty acid content (a proxy for lipid content) was similar in both temperatures. With an increase in cyanobacteria, the total fatty acid content of *Daphnia* decreased linearly at 23°C, while at 20°C the total fatty acid content remained high until cyanobacteria formed >50% of the diet, decreasing after that. This resulted in almost two-fold higher total fatty acid content in *Daphnia* at 20°C compared to 23°C when fed 50% cyanobacteria. Higher temperature resulted in lower fatty acid content of cladocerans in other studies using a much wider temperature gradient 6 to 8°C (Przytulska et al. 2015; Masclaux et al. 2012). This may have important implications for the consumers in higher trophic levels as well. Our results indicate that eutrophication combined with temperature increase may result in a drastically lower *Daphnia* total fatty acid content, PUFA content, and ω-3:ω-6 ratio., which are also important in diets of fish (Sargent et al. 1999, Glencross 2009). Consistent with our results, low ω-3:ω-6 ratio in *Daphnia* indicated poor nutritional status in a previous study (Taipale et al. 2015).

The differences in phytoplankton fatty acid profiles are largely driven by phylogeny, but environmental conditions also have a minor effect (Galloway and Winder 2015). In our data

there are large differences among the algal species in their fatty acid composition, even though small temperature-related variation is also apparent in *Microcystis*. Despite these compositional differences, the total fatty acid content of the algae did not differ among the temperatures, and likely did not cause the lower fatty acid content in *Daphnia* reared at 23°C. Previous studies have found that ω-6 PUFA content in green algae and cyanobacteria increase in lower temperatures (Suschik et al. 2003; von Elert and Fink 2018). Von Elert and Fink (2018) found *Daphnia* growth to increase from 20°C to 25°C, with greater enhancement of growth when the dietary green alga was grown at 25°C, indicating improved food quality of algae grown at a higher temperature. We did not see differences in *Acutodesmus* fatty acids, but the ω-6 PUFA content increased and ω-3 PUFA content decreased in *Microcystis* with temperature, leading to lower ω-3:ω-6 ratios both in the algae and in *Daphnia* feeding on the algae. It is possible that the lack of temperature effects on *Daphnia* size and reproduction could be driven by these changes in algal fatty acids, however, it does not explain why survival of *Daphnia* seemed to increase with higher temperature, although the effect was not statistically significant. Previous studies have found that ω-6 fatty acids may have both positive and negative effects on *Daphnia* growth and reproduction (Becker & Boersma 2005; Martin-Creuzburg et al. 2012; Peltomaa et al. 2017).

We investigated the effect of microplastics on *Daphnia* fitness with low (5%) and high (95%) proportion of cyanobacteria in diet under ambient and elevated temperature. In contrast to our hypothesis, we did not find any effects of the secondary microplastics on *Daphnia* survival, size, or reproduction, even though we used a rather high number of particles (307 000 particles L^{-1} or 0.03 mg C L^{-1}) and stressed the *Daphnia* with low quality food. We were not able to determine the effects of microplastics on fatty acid content when cyanobacteria formed 95% of the diet because of low survival and size of *Daphnia* in those treatments, however, microplastic exposure did not affect total fatty acid content when fed 5%

cyanobacteria. The ingestion of various shapes and sizes of micro- and nanoplastics by *Daphnia* has been confirmed in multiple studies (e.g. Rosenkranz et al. 2009; Besseling et al. 2014; Ogonowski et a. 2016; Jemec et al. 2016; Imhof et al 2017; Rist et al. 2017). We chose to use a mix of secondary microplastics made from household items in our experiment, and thus could not rely on fluorescence for easy quantification of ingestion as other studies have done (e.g. Rosenkranz et al. 2009; Ogonowski et a. 2016; Rist et al. 2017). However, we see no reason why *Daphnia* would not ingest the plastic particles in our study, since they were of similar size to the plastic particles used in other studies. Similar to our results, Imhof et al (2017) found no effects on *Daphnia* survival when they were exposed to a diet consisting of 1% (290,000 particles L^{-1}) secondary microplastics. Several studies have found negative effects of microplastics on *Daphnia*, but only at much higher concentrations/particle numbers than used in our study (Besseling et al. 2014; Ogonowski et al. 2016; Jemec et al. 2016; Martins and Guilhermino 2018). Rist et al. (2017) found no differences in *Daphnia* size or reproduction with an exposure of 1 mg L^{-1} micro- or nanoplastics, and even an exposure concentration of 100 mg L^{-1} did not affect *Daphnia* survival or reproduction in a study by Cannif and Hoang (2018). On the other hand, Sussarellu et al. (2016) found oyster reproduction to decrease when exposed to polystyrene beads at a similar concentration as used in our study (ca. 0.023 mg L^{-1} or 2,000,000 particles L^{-1} or 0.21% of algal biovolume), potentially reflecting differences in the sensitivity between species.

Food quantity also affects the uptake of particles and *Daphnia* performance when exposed to microplastics (Jemec et al. 2016; Rist et al. 2017; Aljaibachi and Callaghan 2018), but we found no interactive effects between food quality and microplastics exposure in *Daphnia*. Furthermore, we found no interactions between temperature and microplastics on *Daphnia*, in contrast to an earlier study that found *Daphnia* sensitivity to primary and secondary microplastics to increase with temperature (Jaikumar et al. 2018). A recent paper highlighted

how the effects of microplastics on organisms are analogous to those caused by other refractory material, such as resuspended sediments (Ogonowski et al. 2018). Thus, the lack of effects is not surprising, since organisms facing other recalcitrant material might have the mechanisms to cope with microplastics, as long as other food – even of poor quality – is also available.

Our findings from this laboratory experiment together with a previous study (Przytulska et al. 2015) imply that increasing proportions of cyanobacteria coupled with a temperature increase due to climate change, may result in fish encountering fewer and smaller *Daphnia*, which have lower fatty acid and PUFA content and produce fewer offspring. However, one should be cautious when predicting outcomes in the field based on small-scale laboratory experiments. Although we aimed at conducting the experiment in an environmentally realistic way, it is impossible to capture the complexity of natural environments in the laboratory. The responses of *Daphnia* to climate change will be caused by dynamic interactions of both abiotic (e.g. temperature, phenology, trophic state) and biotic factors (algal food, predation) (Wojtal-Frankiewicz 2012), and the purpose of the present study was to investigate few of these to isolate their potential effects in a laboratory experiment. Our results are supported by studies in lake Washington and Esthwaite Water, where environmental parameters (i.e. temperature, day length) together with seasonal and interannual differences food quantity and quality (e.g. proportion of edible algae or cyanobacteria) drives the dynamics in *Daphnia* production (George et al. 1990, Scheuerell et al. 2002). However, a mesocosm study investigating how eutrophication (nutrient enrichment) and temperature increase affect plankton community did not find any effects on Cladocera (Özen et al. 2013).

In conclusion, we found that the eutrophication-driven decrease in food quality was more important in determining *Daphnia* fitness than a temperature increase or microplastic exposure. Consistent with other studies using similar exposure concentrations, microplastic

exposure did not have any effects on *Daphnia* fitness. Supporting our results, field studies have also found food quality to be important driver of *Daphnia* production (George et al. 1990, Scheuerell et al. 2002). In the present study, the temperature increase had an interactive effect with food quality that decreased *Daphnia* total fatty acid content almost 50%, which will make them less nutritious prey for higher trophic levels. The drastic negative responses of *Daphnia* populations highlights how anthropogenic eutrophication and climate warming have the potential to shape food-web interactions in aquatic ecosystems.

SUPPLEMENTARY MATERIAL

The Supplementary Material is available online.

AUTHOR CONTRIBUTIONS

Minna Hiltunen: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization, Funding acquisition. Eeva-Riikka Vehniäinen: Conceptualization, Methodology, Writing - Review & Editing. Jussi Kukkonen: Conceptualization, Methodology, Writing - Review & Editing.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tables

Table 1. The average size (mean ± SD) of phytoplankton cells, the mean diameter of microplastic particles, carbon content of phytoplankton and microplastics, and number of particles in microplastic suspensions

	Length/ diameter (µm)	Width (µm)	C (% DW)	Stock suspensions[a] Number (particles mL^{-1})	Stock suspensions[a] Concentration (mg C mL^{-1})	The feeding mix Number (particles mL^{-1})	The feeding mix Concentration (mg C mL^{-1})	*Daphnia* exposure Number (particles L^{-1})	*Daphnia* exposure Concentration (mg C L^{-1})
Phytoplankton									
Acutodesmus sp.	13.3 ± 1.8	6.3 ± 2.2	52						
Nitzschia sp.	19.2 ± 2.4	5.4 ± 1.3	28						
Microcystis aeruginosa	3.9 ± 1.0		46						
Microplastics									
ABS	3.2		84	1 844 000	0.155	218 000	0.018	119 000	0.010
PS	3.7		90	4 525 000	0.054	134 000	0.018	73 000	0.010
PET	3.7		61	398 900	0.392	211 000	0.018	115 000	0.010

[a] The numbers of microplastic particles in stock suspensions was measured with a particle counter (rounded to nearest 1000), and carbon concentration was calculated based on literature values of density and measured values of carbon content (see methods for details).

Table 2. *Daphnia* diet treatments were run in 20°C and 23°C and consisted of 4 replicates with 10 *Daphnia* neonates in each. *Daphnia* were exposed to gradient of cyanobacteria from 5 to 95% of the diet simulating eutrophication in two temperatures (20 and 23°C). The treatments with 5 and 95% cyanobacteria were conducted with and without microplastic exposure (1% of the diet).

	Proportion of diet (%)			
Treatment	*Microcystis*	*Nitzschia*	*Acutodesmus*	MPs
5% cyano	5.0	47.5	47.5	-
25% cyano	25.0	37.5	37.5	-
50% cyano	50.0	25.0	25.0	-
75% cyano	75.0	12.5	12.5	-
95% cyano	95.0	2.5	2.5	-
5% cyano + MPs	5.0	47.0	47.0	1.0
95% cyano + MPs	95.0	2.0	2.0	1.0

Table 3. Results from generalized linear model testing for differences in *Daphnia* survival and reproduction in relation to proportion of cyanobacteria in diet and temperature. *Daphnia* were exposed to gradient of cyanobacteria from 5 to 95% of the diet simulating eutrophication in two temperatures (20 and 23°C).

Model	Wald χ^2	df
Survival		
Cyano in diet (%)	25.691**	1
Temp	1.373	1
Cyano in diet (%) * temp	2.949	1
Neonates per surviving adult		
Cyano in diet (%)	580.66**	1
Temp	0.191	1
Cyano in diet (%) * temp	2.927	1

* < 0.01
** < 0.001

Table 4. Results from a general linear model testing for differences in *Daphnia* fitness parameters in relation to proportion of cyanobacteria in diet and temperature. *Daphnia* were exposed to gradient of cyanobacteria from 5 to 95% of the diet simulating eutrophication in two temperatures (20 and 23°C).

Model	*SS*	*df*	*F*
Length at 6 days[a]			
Cyano in diet (%)	0.125	4	62.783**
Temp	0.001	1	1.945
Cyano in diet (%) * temp	0.000	4	0.189
Length at 14 days			
Cyano in diet (%)	13.645	4	124.603**
Temp	0.018	1	0.647
Cyano in diet (%) * temp	0.051	4	0.466
Total FA content			
Cyano in diet (%)	3264.568	3	10.994*
Temp	1423.997	1	14.386*
Cyano in diet (%) * temp	3355.509	3	11.300*
ω-3:ω-6 ratio			
Cyano in diet (%)	80.108	3	160.357**
Temp	31.438	1	188.793**
Cyano in diet (%) * temp	2.508	3	5.020*

[a] length at 6 days was log (x+1) transformed prior analysis.

* < 0.01

** < 0.001

Figures

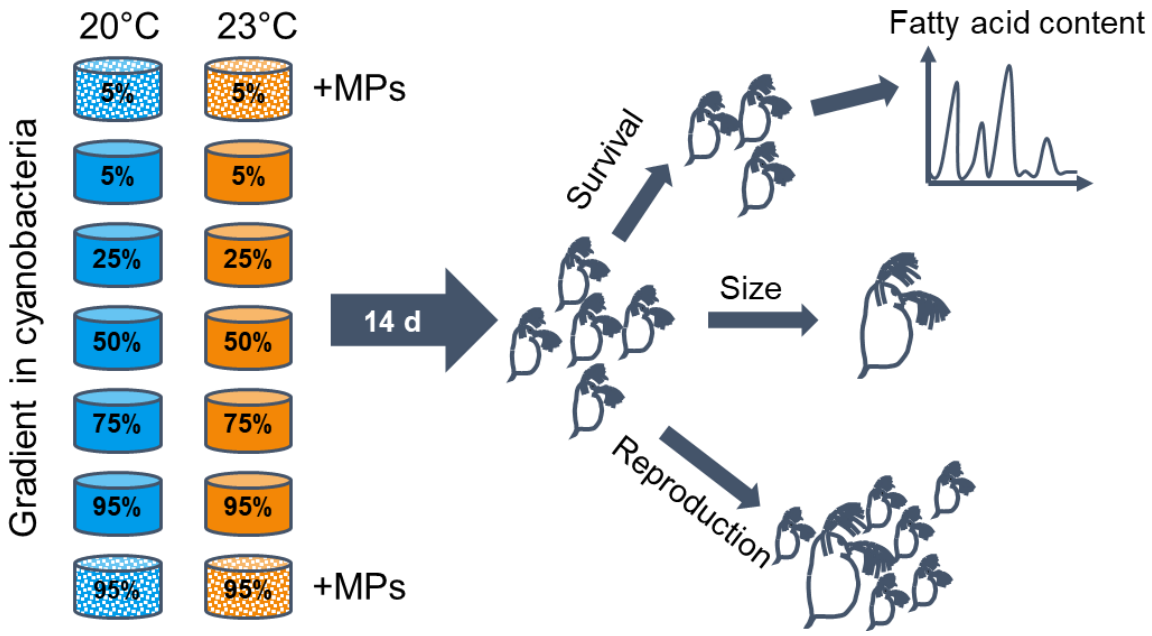

Figure 1. A schematic diagram of the experimental design. *Daphnia* were exposed to gradient of cyanobacteria from 5 to 95% of the diet simulating eutrophication in two temperatures (20 and 23°C). The treatments with 5 and 95% cyanobacteria were conducted with and without microplastic exposure (1% of the diet).

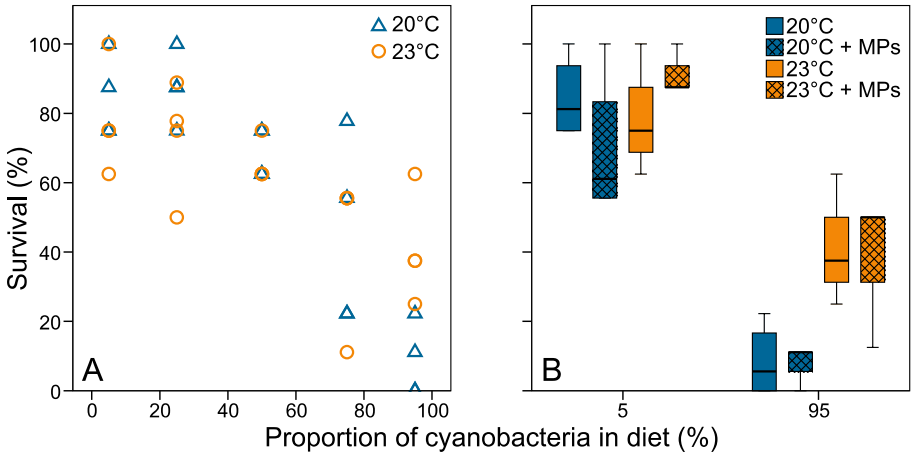

Figure 2. Survival of *Daphnia* A) in relation to proportion of cyanobacteria in the diet and temperature, B) survival with and without microplastic (MP) exposure when fed 5% or 95% cyanobacteria. The box represents the 25th and the top 75th quartile, while the line is the median. The whiskers represent the maximum and minimum values. *Daphnia* were exposed to gradient of cyanobacteria from 5 to 95% of the diet simulating eutrophication in two temperatures (20 and 23°C). The treatments with 5 and 95% cyanobacteria were conducted with and without microplastic exposure (1% of the diet).

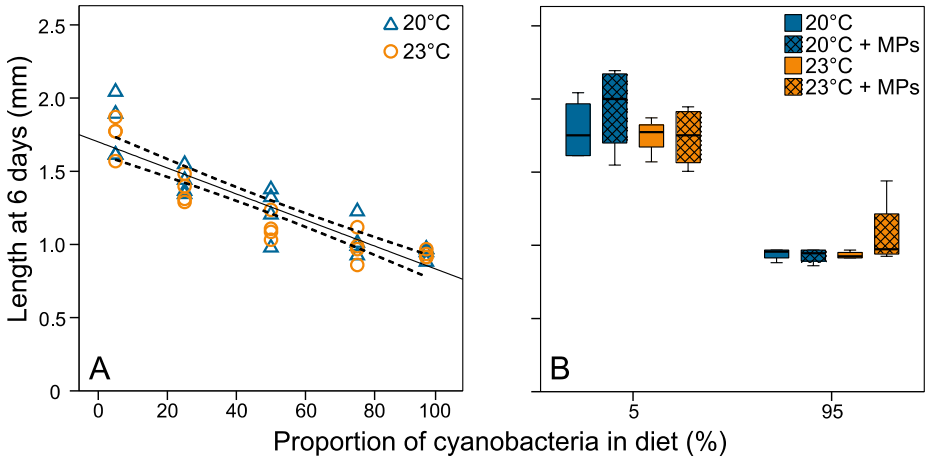

Figure 3. Length of six-day-old *Daphnia* at 20°C or 23°C A) with diets varying in proportion of cyanobacteria. Line represents linear regression with 95% confidence intervals (y = -0.009x +1.701, R^2=0.817). B) with and without microplastic (MP) exposure in 20°C and 23°C with diets of 5% and 95% cyanobacteria. *Daphnia* were exposed to gradient of cyanobacteria from 5 to 95% of the diet simulating eutrophication in two temperatures (20 and 23°C). The treatments with 5 and 95% cyanobacteria were conducted with and without microplastic exposure (1% of the diet).

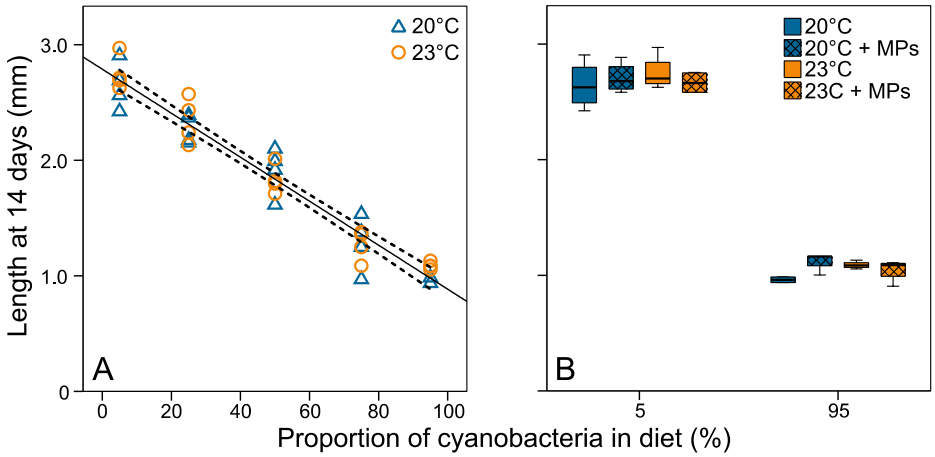

Figure 4. Final length of 14-day-old *Daphnia* at 20°C or 23°C A) with diets varying in proportion of cyanobacteria. Lines represent linear regression with 95% confidence intervals (y = -0.019x+2.788, R^2 = 0.937), B) with and without microplastic (MP) exposure in 20°C and 23°C with diets of 5% and 95% cyanobacteria. *Daphnia* were exposed to gradient of cyanobacteria from 5 to 95% of the diet simulating eutrophication in two temperatures (20 and 23°C). The treatments with 5 and 95% cyanobacteria were conducted with and without microplastic exposure (1% of the diet).

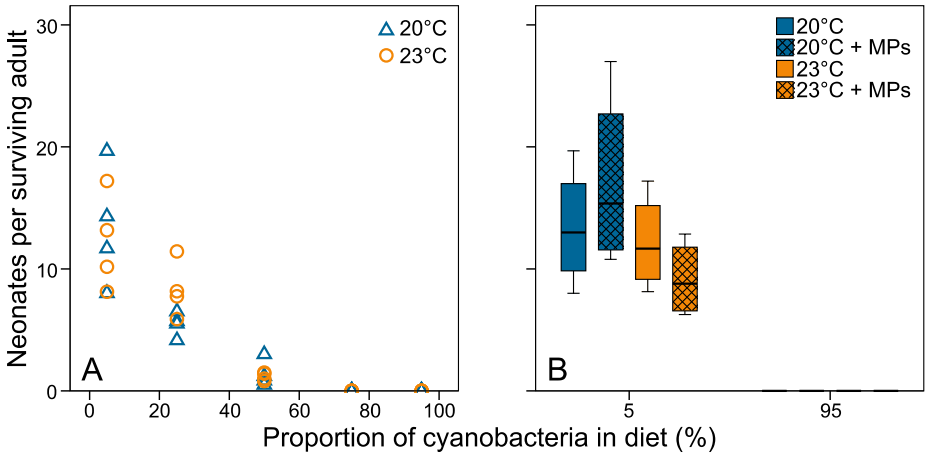

Figure 5. Neonates per surviving adult A) with *Daphnia* grown at 20°C or 23°C with diets varying in proportion of cyanobacteria, B) with and without microplastic (MP) exposure in 20°C and 23°C with diets of 5% and 95% cyanobacteria. *Daphnia* did not produce any offspring when fed 95% cyanobacteria. *Daphnia* were exposed to gradient of cyanobacteria from 5 to 95% of the diet simulating eutrophication in two temperatures (20 and 23°C). The treatments with 5 and 95% cyanobacteria were conducted with and without microplastic exposure (1% of the diet).

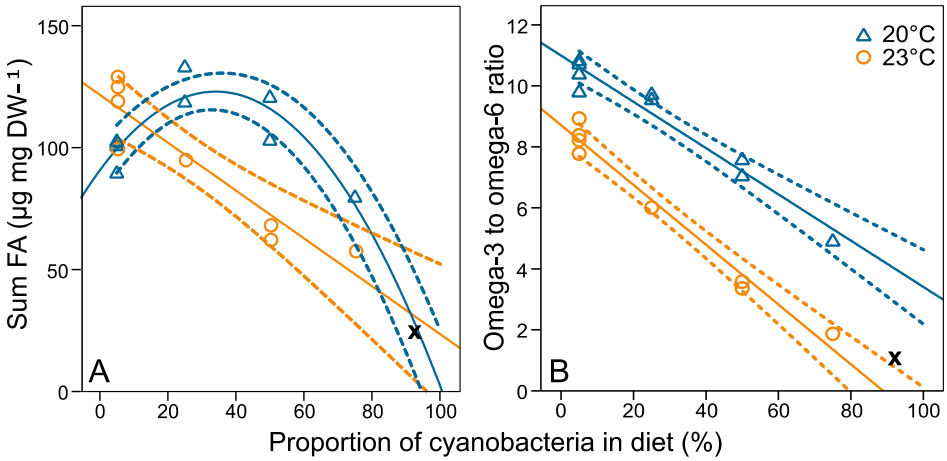

Figure 6. Regression between the proportion of cyanobacteria in the diet and A) the total fatty acid content (sum FA) of *Daphnia* for (23°C: linear, y = -0.966x +119.530, R^2 = 0.872 and for 20°C: 2nd order polynomial, y = -0.028x^2 +1.894x + 90.638, R^2= 0.779). B) ω-3:ω-6 ratio of *Daphnia*. Linear regression for 20°C (y = -0.076x +10.995, R^2 = 0.948) and for 23°C (y = -0.098x +8.712, R^2 = 0.975). The marker x represents *Daphnia* fed 95% cyanobacteria, which was analyzed from a pooled sample consisting of both temperatures with and without microplastics (value not included in regression models). *Daphnia* were exposed to gradient of cyanobacteria from 5 to 95% of the diet simulating eutrophication in two temperatures (20 and 23°C). The treatments with 5 and 95% cyanobacteria were conducted with and without microplastic exposure (1% of the diet).

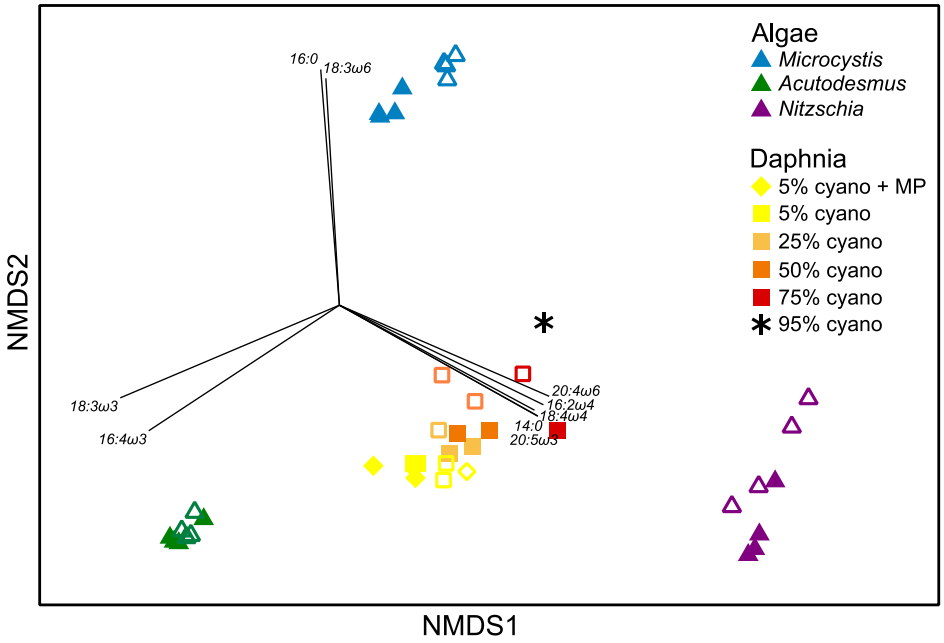

Figure 7. Non-metric multidimensional scaling (NMDS) ordination of the percent fatty acid composition of algae and *Daphnia* in the experiment. The stress for the 2-D solution was 0.04. The fatty acids that most strongly correlate with the axes ($r > 0.9$) are presented as vectors. Filled symbols represent phytoplankton or *Daphnia* that were raised in 20°C and open symbols represent those raised in 23°C. *Daphnia* in all the treatments with 95% cyanobacteria (both temperatures and with/without microplastics (MPs)) were combined to gain enough material for fatty acid analysis. *Daphnia* were exposed to gradient of cyanobacteria from 5 to 95% of the diet simulating eutrophication in two temperatures (20 and 23°C). The treatments with 5 and 95% cyanobacteria were conducted with and without microplastic exposure (1% of the diet).

Graphical abstract caption: *Daphnia magna* were grown along a gradient in cyanobacteria (5-95%, mimicking eutrophication) in ambient and +3°C temperature with and without exposure to a mix of secondary microplastics (MPs). Proportion of cyanobacteria in diet was the major driver of *Daphnia* fitness, and had minor interactions with temperature, while we found no effects of MPs.