The effects of anthropogenic stressors on mercury concentrations and community composition of freshwater zooplankton

by

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A thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science
in
Environmental Science and Management

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Portland State University
2016

Abstract

Methylmercury (MeHg) bioaccumulation in freshwater aquatic systems is impacted by anthropogenic stressors, including climate change and excess nutrients. The goal of this study was to determine how warmer water temperatures and excess nutrients would impact zooplankton communities and phytoplankton concentrations, and in turn increase or decrease MeHg concentrations in freshwater zooplankton. I used a 2x2 factorial design to determine if the interaction of temperature and nutrients would impact plankton metrics and zooplankton MeHg concentrations. Mesocosms were filled with Hg-contaminated water and plankton from Cottage Grove Reservoir, Oregon, U.S.A, a waterbody that has experienced decades of elevated MeHg concentrations and corresponding fish consumption advisories due to run-off from Black Butte Mine tailings, located within the watershed. Treatment combinations of warmer temperature (increased by 0.5°C) and nutrient addition (a single pulse of excess nitrogen and phosphorous), control, and a combination of temperature and nutrients were applied to mesocosms. While plankton did respond to treatments, zooplankton biomass and phytoplankton concentrations did not have significant relationships to MeHg concentrations. However, a significant interactive effect of nutrients and temperature was present: nutrients appeared to buffer against increased MeHg concentrations when temperature was elevated. The mechanisms for this interaction appear to be related to a shift to larger body size and an increase in abundance of *Daphnia* over copepods. Findings suggest that community composition and species-specific differences in both zooplankton and phytoplankton could play a role in MeHg transfer to higher trophic levels.

Dedication

For my daughter, Mia, whose patience, humor and optimism made grad school possible.

Acknowledgements

I am indebted to all members of the Strecker Aquatic Ecology Lab at Portland State University for their help, kindness, humor and assistance: Angela Strecker, Sam Cimino, Jeff Brittain,Tim Elder, Ari Chiapella, Crysta Gantz, Amelia Johnson, Brian McGann and Meredith Holgerson. Much gratitude to Gene Foster and Joe Maser for their time, feedback and support as committee members. Many thanks to fellow students Joey Peters, Monica Mogilewsky, Alex Staunch and Kit Rouhe for encouragement and moral support. This project was not doable without the assistance of field and lab assistants Jared Anderson and Jesse Klinger, nor without the wisdom and guidance of Rich Miller and Steve Wells from the Center for Lakes and Reservoirs. Thanks to Sean Dinsmore and Zachary Neumann for sample assistance. Many thanks to Stewart Rounds of USGS and Chris Deutsch of PSU for freeze-drying assistance, and to the CCAL Lab at OSU for nutrient sample analysis. Huge thanks to Robin Stewart and Collin Eagles-Smith of USGS for mercury analysis, patience and assistance in interpreting data. Thanks to Chris Eckley for providing insights and ideas on experimental design. Mark Chappelle, Kathryn Tackley and Dan Turner of the US Army Corps of Engineers were instrumental in site scouting and set-up. Many thanks to Sigma Xi and the local Willamette Chapter of Sigma Xi for funding support. Last, thanks to the infinite kindness of my friends and family for countless pep talks, encouragement and love throughout this experience.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

Chapter 1: **Background**

Mercury (Hg) in aquatic systems is a well-documented hazard to human health due to its behavior as a lipophilic, potent neurotoxin at particularly small doses (Morel et al. 1998, Mergler et al. 2007). The widespread presence of this toxin in freshwater and marine systems, through both anthropogenic and naturally occurring pathways, is a human health hazard due to possible Hg exposure through fish consumption (Mergler et al. 2007). Inorganic mercury becomes more toxic in water as a combination of anoxic conditions, certain key bacteria, and adequate carbonaceous material (e.g., detritus, decaying organisms) lead to mercury methylation, which creates the more toxic and bioavailable Hg species, methylmercury (MeHg) (Watras and Bloom, 1992, Chen and Folt 2005, Parks et al. 2013).

Once mercury has methylated in an aquatic system, it remains there through resuspension, and readily bioaccumulates up the food chain, becoming more concentrated at each trophic level (Watras and Bloom 1992, Mason et al. 1994, Watras et al. 1998, Stewart et al. 2008, Gantner et al. 2009). While other metals can accumulate in aquatic organisms through water column concentrations via respiration (i.e., through the gills), the primary pathway for MeHg bioaccumulation appears to be dietary (Eagles-Smith et al. 2008, Stewart et al. 2008). From one trophic level to the next, there can be up to a 10-fold increase in tissue concentration of mercury via the process of bioaccumulation (Morel et al. 1998). For example, fish, eating zooplankton that consume phytoplankton with MeHg concentrations as small as 1 $ng \cdot g^{-1}$, could ultimately develop fish tissue concentrations of 100$ug \cdot g^{-1}$ because of MeHg bioaccumulation (Morel et al. 1998). In

particular, the ability of methylmercury to adsorb to sediments appears to be central to its persistence and the ease with which it transfers from one trophic level to the next (Ramalhosa 2006). The end result is that the largest, oldest fish have the highest concentrations of MeHg, hence there are frequently fish consumption advisories against top predator fish like blue-fin tuna (Lowenstein et al. 2010). Similarly, top predators in freshwater systems contaminated with Hg are most likely to have toxic levels of MeHg (Hammerschmidt and Fitzgerald 2006).

On a local level, mercury is a pollutant of concern for the state of Oregon, as waterbodies here receive Hg inputs from mining activities as well as significant atmospheric inputs from long-range transport across the Pacific Ocean (Jaffe et al. 2005, ODEQ 2006). The Oregon Department of Environmental Quality (ODEQ) has recently revised their per-capita fish consumption rate (from 17.5 to 175 $g \cdot d^{-1}$) to better protect the health of subsistence fishing communities who consume, on average, much more fish than the previous limit (ODEQ 2011). The implication of this revision is that non-point source pollutants (primarily from run-off, but atmospheric deposition as well) must be reduced further to meet these more stringent standards. Achieving this reduction in mercury inputs is particularly challenging, because although Hg can get into waterbodies directly via runoff, atmospheric deposition of mercury from other distant countries (such as China and India) can contribute greatly to localized Hg concentrations (Jaffe et al. 2005, Hammerschimdt and Fitzgerald 2006, Gantner et al. 2010). Based on 2012 sampling, median MeHg fish tissue concentrations across U.S. streams and rivers were 0.33 $mg \cdot kg^{-1}$ (Wentz et al. 2014). Even in freshwaters without direct point sources of

mercury, a baseline level of the contaminant is evident throughout the country (Wentz et al. 2014).

How mercury gets into freshwater systems

Mercury enters waterbodies through a series of possible pathways. First, mercury is directly input from anthropogenic activities, such as mercury amalgamation runoff from gold processing (Ambers and Hygelund 2001) or effluent from pulp mills (Mason et al. 1994). Second, mercury enters waterbodies through indirect anthropogenic inputs, including atmospheric deposition from coal-burning power plants and chlor-alkali plants (Morel et al. 1998, Jaffe et al. 2005, Dufault et al. 2009). Indirect, anthropogenic pathways are in fact a greater source of mercury globally compared to direct pathways (Driscoll et al. 2013). Third, mercury can also contaminate waterbodies through naturally occurring inputs like cinnabar, which occurs in seams in local geology, and when exposed to the elements (from mining or other geologic disturbance), becomes a significant source of Hg (Ambers and Hygelund 2001). Mercury-rich runoff coming from mine tailings and exposed and abandoned mining sites mobilizes through a watershed after precipitation events, through downslope soil erosion, runoff and flooding, ultimately ending up in reservoirs and lakes (Curtis et al. 2013). Natural events like volcanic activity and forest fires are also considered to be a significant, though sporadic and much smaller, source of atmospheric Hg deposition to waterbodies (Mason et al. 1994).

The effects of temperature on mercury in freshwater systems

Once mercury enters a waterbody, abiotic factors, such as temperature, can influence the rate of Hg bioaccumulation. Methylation of inorganic mercury primarily

occurs in the sediments of freshwater systems, where sulfate and iron-reducing bacteria convert the metal into the more bioavailable and toxic form, methylmercury (Fleming et al. 2006). Studies have found higher rates of sediment methylation in warmer water temperatures, and numerous observations of mercury-contaminated systems have found that methylmercury concentrations in the sediment peak during late summer, when water temperatures are highest (Winfrey and Rudd 1990, Mauro et al. 1999). As MeHg moves up the food web, temperature continues to influence the rate at which organisms bioaccumulate mercury. The literature indicates that the influence of metal toxicity increases with a rise in temperature in many cases, primarily due to an increase of an organism's metabolic rate in warmer conditions. Increased respiratory rates require more energy to maintain, so organisms consume more at these warmer temperatures, resulting in greater metal exposure (DeMott 1982, Huegens et al. 2005, Gutierrez et al. 2012). Both laboratory and field experiments showed significant increases in MeHg bioaccumulation when marine killifish were exposed to warmer water temperatures (Dijkstra et al. 2013). Much research has been done on other metals (e.g., zinc, chromium and selenium) and how temperature magnifies the toxicity of these pollutants (Moore and Folt 1993, Huegens et al. 2006), but there is a gap in the literature on how methylmercury concentrations in freshwater organisms (zooplankton in particular) change when temperatures increase. Given that a possible air temperature increase in the Pacific Northwest of the United States of 3-4.5°C by 2080 is feasible, climate change must be considered as a factor in how methylmercury concentrations in freshwater organisms might change in the foreseeable future (Solomon 2007, Mote and Salathé 2010).

The effects of nutrients on mercury in freshwater systems

The conditions needed for mercury to methylate and bioaccumulate are often supported by the addition of excess nutrients in aquatic systems. Nutrient pulses can serve as growth catalysts for all algae, including both sulfate-reducing bacteria and cyanobacteria, creating low oxygen conditions when the algae die. This combination of anoxic conditions and high primary production create an ideal environment for Hg methylation, which can explain why wetland areas are often the greatest producers of MeHg (Pickhardt et al. 2005, Morel et al. 1998, Chen et al. 2005). It has been fairly recently established, however, that an increase in lake primary productivity appears to mitigate MeHg bioaccumulation to higher trophic levels (Pickhardt et al. 2002, Chen et al. 2005, Chen and Folt 2005). Despite in many cases having shorter food chains, oligotrophic and otherwise pristine lakes have shown a higher rate of MeHg accumulation than eutrophic lakes (Chen et al. 2005, Gantner et al. 2010). The mechanism for this mitigation appears to be biodilution; there are simply more phytoplankton to take up the MeHg in eutrophic systems, and so zooplankton and fish that feed on these phytoplankton are taking in less MeHg per unit than they would in less productive oligotrophic systems (Pickhardt et al. 2002).

The role of zooplankton community composition on MeHg concentrations

While warming temperatures and excess nutrients can have direct effects on MeHg concentrations in zooplankton, shifts in zooplankton community, caused by said stressors, can also impact the availability of MeHg to higher trophic levels. Chen and Folt (2005) determined that higher total zooplankton abundance appeared to dilute MeHg concentrations in fish; however, other studies have found that additional factors such as seasonal differences in species composition and dominance could explain MeHg

concentration differences (Watras and Bloom 1992, Kuwabara et al. 2006). Other research indicates that species identity is not as critical as larger overall plankton body size when estimating potential MeHg concentrations at the base of the food web (Kainz et al. 2006). Others have found that particular zooplankton orders, such as copepods or cladocerans, could have an effect on total MeHg concentrations as a function of their different feeding, reproductive and metabolic rates; generally, copepods appear to bioaccumulate MeHg less efficiently than cladocerans (Stewart et al. 2008, Pickhardt et al. 2005).

The objectives for my thesis were to examine potential interactive effects of increased temperature and nutrient addition on methylmercury concentrations in zooplankton. My goal was to determine if these common stressors would impact zooplankton communities, and thus change mercury concentrations both directly (e.g., a change in species abundance impacting Hg concentrations) and indirectly (e.g., lower Hg concentrations in more productive systems resulting from higher nutrient concentrations). I hypothesized that the zooplankton and phytoplankton communities would act as "middle men" between the effects of warmer temperatures and added nutrients. Specifically, I predicted that temperature would decrease zooplankton biomass, and increase the ratio of cladocerans to copepods, resulting in higher zooplankton MeHg concentrations, and that nutrients would increase phytoplankton biomass (using chlorophyll *a* as a proxy) and consequently lower zooplankton MeHg concentrations (Figure 1). This study used a 2x2 factorial design mesocosm experiment to determine the interactive effects of warming and nutrient addition on zooplankton community composition and zooplankton mercury concentrations using organisms and water from

Cottage Grove Reservoir, an Hg-contaminated Superfund site in Central Oregon, U.S.A. (Figure 2, 3). I predicted that in the combination treatment of warming and nutrients, the algal biodilution effect would ultimately mitigate the potential for increased mercury concentrations caused by increased temperature, as phytoplankton would respond positively to the interactive combination of warmer temperatures and excessive nutrients. I anticipated that the warming treatment would have the highest MeHg zooplankton concentrations, the nutrient treatment the lowest concentrations, and that the resulting changes in zooplankton community due to treatments could ultimately explain some of these differences in MeHg concentrations. Given the persistence of mercury in aquatic systems, the results of this experiment could add valuable information on how common stressors will impact this neurotoxin, and ultimately, how these stressors might change MeHg concentrations in higher trophic levels.

Chapter 2: Study

Freshwater systems now bear the burdens of human-induced stressors to a greater extent than previously historically recorded. Atmospheric inputs of persistent pollutants like mercury (Hg) alter aquatic systems and pose human health risks through food web bioaccumulation (Meybeck and Vörösmarty 2005, Mergler et al. 2007). Critically, other anthropogenic stressors, such as climate change and eutrophication, can alter Hg methylation and subsequent bioaccumulation, which has consequences for human consumption of large-bodied, top predator fishes (Pickhardt et al. 2002, Ficke et al. 2007). As Hg is primarily accumulated through a dietary pathway, it is critical to understand how freshwater organisms mediate the transfer of Hg to the highest trophic levels (Fitzgerald and Mason, 1997, Kuwabara et al. 2006, Eagles-Smith et al. 2008, Stewart et al. 2008). Despite the potential impacts to human and aquatic ecosystem health, the effects of anthropogenic stressors on Hg bioaccumulation in freshwater food webs have not been extensively examined (Smith et al. 1998, Booth and Zeller 2005).

The cascading effects of climate change on freshwater systems are already well underway. Global average temperatures have increased by 0.8°C since the Industrial Revolution of the mid-1800s (Hartmann et al. 2013). Warmer temperatures have resulted in impacts to many aquatic species, beginning at the base of the food web (Ficke et al. 2007). Increased temperatures have shifted phytoplankton communities such that less edible or toxic species thrive, limiting energy transfer to higher trophic levels due to an increase in lower quality food (Butler et al. 1989, Paerl et al. 2011). Warmer temperatures have caused the loss of cohorts of zooplankton, resulting in altered communities, including loss of diversity, decreases in body size, and reduced fecundity (Moore and

Folt, 1993, Chen and Folt 1996, Weetman and Atkinson 2004). These community shifts can impact higher level consumers through changes in food quality and availability (Chen and Folt 1996). Lastly, climate change and metal bioaccumulation appear to be linked, as warmer water temperatures have been shown to increase the metabolic rates of fishes, causing them to feed at higher rates, and thus bioaccumulate metals faster as compared to fish in cooler water temperatures (Djikstra et al. 2013).

The exponential rise of modern agriculture since the Industrial Revolution has also impacted aquatic systems. Nutrient-rich runoff from agricultural areas has changed aquatic food-web dynamics due to eutrophication and shifts in primary productivity (Smith et al. 1998, Ramankutty and Foley 1999). One such shift has included an increase in algal blooms due to excessive amounts of nitrogen and phosphorus in agricultural runoff. Many of the phytoplankton species stimulated by excess nutrients are largely inedible to freshwater zooplankton, thus excess nutrients can impact both food availability and quality (Vitousek et al, 1997, Correll 1998, Brett et al 2000, Pearl et al. 2011). These algal blooms also tend to result in higher phytoplankton biomass as compared to more oligotrophic systems that do not receive these nutrient-rich inputs (Heisler et al. 2008, Smith and Schindler 2009).

This increase in nutrient-induced primary productivity has ramifications for how metals, mercury in particular, move through aquatic food webs. There is still ambiguity in whether highly productive systems will contribute to mercury methylation via a supply of increased carbonaceous material, or whether such eutrophic systems will buffer higher trophic levels from mercury uptake due to biodilution. Biodilution occurs when a proliferation of algae dilutes available MeHg before it can get to higher consumers

(Pickhardt et al. 2002, Chen and Folt 2005). Whether Hg will methylate readily or not is system-dependent: while wetlands are MeHg producers, and reservoirs are seasonal MeHg producers, temperature and primary productivity seem to be key factors in this process (Figure 1) (Eagles-Smith et al. 2008, Stewart et al. 2008, Djikstra et al. 2013). In mercury-contaminated waterbodies, understanding the factors that might control mercury methylation can assist with management decisions if MeHg bioaccumulation poses human health risks.

Nutrient loading and warmer temperatures in aquatic systems can impact MeHg concentrations directly, as well as cause shifts in zooplankton community composition, which may indirectly result in changes to MeHg concentrations in higher level predators (Winder et al. 2009). High zooplankton abundance can dilute MeHg concentrations in fish (Chen and Folt 2005), but other studies have found that seasonal differences in species composition and dominance confound the biodilution hypothesis (Watras and Bloom 1992, Kuwabara et al. 2006). Ideas as to the precise mechanisms that promote MeHg bioaccumulation differ. Kainz et al. (2006) found in their research on zooplankton essential fatty acids that larger zooplankton body size, not identity, was critical in estimating potential MeHg concentrations at this trophic level. In contrast, others have found that particular zooplankton orders, such as copepods or cladocerans, could have an effect on total MeHg concentrations as a function of their different feeding, reproductive, and metabolic rates; cladocerans generally show higher MeHg concentrations than copepods, despite having lower trophic positions in most cases (Stewart et al. 2008, Pickhardt et al. 2005, Rennie et al. 2011). Even ontogeny of zooplankton species can have ramifications for MeHg bioaccumulation: as lipid content changed over the lifespan

in the copepod, *Limnocalanus macrurus*, so too did MeHg concentrations (Chételat et al. 2012). Zooplankton community metrics, and their drivers, are clearly complex, and an important consideration in the movement of MeHg through an aquatic food web.

Project purpose and hypotheses

The purpose of this study was to examine the possible effects of two common stressors, increased temperature and nutrients, on both the community composition and the mercury concentrations of zooplankton using organisms from a mercury-contaminated reservoir. My questions were: (1) Would the combination of these stressors mitigate mercury concentrations through shifts in zooplankton community composition, abundance, and life history? (2) Would warming water cause zooplankton to take up more mercury, as is the case with other toxic metals? (3) Would nutrients buffer that uptake by zooplankton via absorption of mercury by increased densities of phytoplankton?

I hypothesized that: (1) zooplankton community shifts (e.g., increases in cladocerans, decreases in copepods) and decreases in biomass resulting from higher temperature will increase zooplankton MeHg concentrations, while nutrients would decrease MeHg concentrations (Figure 2) (Moss et al. 2011); (2) warming water will show the highest mercury concentrations in zooplankton because of the increased metabolic rate of zooplankton (using body size and egg counts as proxies of metabolism) (Dijkstra et al. 2013); (3) nutrient-rich, and therefore more productive systems, will show the lowest concentrations of mercury in zooplankton because of the biodilution effect

(using chlorophyll *a* concentrations as a proxy for phytoplankton densities) (Pickhardt et al. 2002, Chen et al. 2005, Chen and Folt 2005); (4) when nutrients and warmer water are both present, the biodilution effect will ultimately mitigate the potential for increased mercury concentrations caused by increased temperature, as phytoplankton densities would increase in response to the interactive combination of warmer temperatures and excessive nutrients (Moss et al. 2011).

Methods

Study site

The experiment took place at Cottage Grove Reservoir, nearly five miles south of Cottage Grove, Oregon (latitude: 43°43'00", longitude: 123°02'55") (Figure 3). The reservoir is located at river mile 29 of the Coast Fork of the Willamette River. The reservoir resides in the same watershed as the Black Butte Mine, where cinnabar mining and abandoned mine tailings have led to elevated total and methylmercury levels in fish tissue, prompting a nearly continuous fish consumption advisory for the reservoir since 1979, nearly ten years after the mine closed (Curtis et al. 2013). In the mainstem of the Willamette River in Oregon, fish tissue MeHg concentrations averaged 0.47 $mg \cdot kg^{-1}$, while fish tissue concentrations in Cottage Grove Reservoir, upstream of the mainstem, averaged 1.63 $mg \cdot kg^{-1}$ (Hope and Rubin 2005). To address these elevated mercury concentrations, the US Environmental Protection Agency listed the Black Butte Mine area as a Superfund site in 2010. Currently, the dam and subsequent management of the reservoir serves primarily as local flood reduction; however the reservoir also acts as a recreational, irrigation and downstream navigation resource.

Experimental set-up

To test my hypotheses, I used a 2x2 factorial design with two treatments (temperature and nutrients), each with two levels (with and without), with four replicates per treatment combination. Treatments were assigned randomly to sixteen, grey 379-L cattle watering tanks (132.08cm x 78.11cm x 60.96cm; High Country Plastics, Caldwell, ID). Experimental set-up occurred on the eastern shore of the reservoir. The experimental site was chosen for proximity to the reservoir, security and its distance from public use. Reservoir water was pumped into a storage tank from a depth of one meter at the boat ramp on 12 July 2013. Water was then pumped into the tanks after filtering through 10-µm nylon mesh to remove large sediments and screen out large phytoplankton and zooplankton. Although sediment methylation of mercury is generally the source of MeHg in freshwater systems, I determined that adding reservoir sediments to the mesocosms (with unknown and unstandardized metal concentrations) could have confounded my results. I performed a pilot study prior to experiment start and found that zooplankton from Cottage Grove Reservoir had greatly elevated MeHg levels (Eagles-Smith, personal communication). Given the short term nature of my experiment, I determined that the amount of MeHg already present in zooplankton would be sufficient to see treatment effects, if any were present. On 16 July 2013, tanks were inoculated with reservoir phytoplankton and zooplankton, collected by vertical tows from the reservoir's epilimnion (0-4m) using a 30-cm diameter plankton net with 80-µm mesh. Plankton were transported using 5-L carboys filled to capacity with unfiltered reservoir water, and deposited into tanks promptly after collection. Reservoir zooplankton density was estimated by sampling the reservoir at five locations at depths of 3 meters, the shallowest

depth where both adequate zooplankton mass for MeHg analysis was available as well as the closest depth comparable to the depth of the tanks. A subsample of zooplankton was counted from each site, and then counts were averaged across all five sites into one composite estimate of zooplankton density. To compensate for variance and potential loss of plankton to stress of collection and transport, tanks were inoculated at 1.5x the ambient reservoir zooplankton density. Mosquito larvae and mites were removed by hand. All tanks were then covered with mosquito netting to minimize mosquito breeding and other invertebrate colonization within the tanks, and left to equilibrate before treatments began.

On 18 July 2013, the temperature treatment was applied using custom-built passive greenhouse canopies as per Strecker et al. (2004) (Figure 4). Greenhouse canopies were used to passively warm temperature treatment tanks as compared to control tank water temperatures by approximately 0.5°C, a conservative and near-future representation of climate change in the Pacific Northwest (Mote and Salathé 2010). Canopies were constructed using ¾" PVC pipe, Tufflite IV greenhouse sheeting (6 mil thickness), plastic louvered dryer vents and marine-grade staples to minimize potential wear and rust for the duration of the experiment. All tanks were covered by these canopies to control for solar radiation. Temperature treatments had canopies lowered to sit on edge of tanks, and all vents were closed. Control tanks had canopies raised approximately 25.4 cm off of tank edge, and all vents were opened. At each weekly temperature sampling, vents were closed or opened to adjust for desired temperature based on treatment. Canopies were held in place by rope tied to rebar supports on the perimeter of each tank.

Nutrient treatments were also applied on 18 July 2013. Nutrient treatment tanks received a single addition of nitrogen, as KNO_3, and phosphorous, as KH_2PO_4, at amounts equaling a ten-fold increase over ambient reservoir levels of total nitrogen and total phosphorous, 0.19 $mg \cdot L^{-1}$ and 0.013 $mg \cdot L^{-1}$, respectively (USACE, unpublished data). This pulse of nutrients was intended to replicate a nutrient-loading event at levels high enough to increase productivity to eutrophic levels from the reservoir's typically mesotrophic conditions (Wetzel 2001). The tanks were stirred manually to distribute nutrients; all tanks were stirred to control for any unintended effects caused by the water disturbance.

Sampling and sample processing

Generally, sampling of the reservoir and the experimental tanks occurred weekly, beginning 18 July 2013 (day 0) to 22 August 2013 (day 35). Day 0 sampling took place before treatments were applied. Zooplankton community samples were collected weekly in the reservoir by vertical plankton tows using a 30-cm diameter plankton net with 80-µm mesh from 3 m above the lake bottom to water surface. Mesocosm zooplankton samples were collected by taking a 22-L water sample with a Van Dorn sampler, followed by filtration with 80-µm mesh. Water was returned to mesocosms after zooplankton were filtered out. Zooplankton samples were stored at a final solution of 70% ethanol. Zooplankton abundance was then calculated by splitting each sample into fractions and counting at least 250 individuals, with a minimum of 50 for each species, and no more than 50 copepodids or 30 nauplii per order (Strecker and Arnott 2005). Zooplankton eggs and body size were used as possible metrics of changes in metabolism (Orcutt and Porter 1984). Eggs were sorted by the major zooplankton groups of

cladocerans and copepods and were counted for as many sample fractions as were needed to reach adequate adult zooplankton counts. Total egg counts were divided by abundance of either cladocerans and copepods to obtain a standardized metric of eggs per individual. Zooplankton counts and identification were made using a Leica M165C microscope and IC80HD camera (Leica Microsystems Inc., Buffalo Grove, IL). Taxonomic keys were used to identify adults to species level where possible; juveniles were identified to order or subclass (Thorp and Covich 2009, Haney et al. 2013). Body lengths of a subsample of 10 zooplankton from each species from each tank for all five weeks were measured and averaged. Length-weight regressions were used to estimate biomass by using the averaged length of 10 individuals per taxa per sample (McCauley 1984, Culver et al. 1985, Lawrence et al. 1987).

Zooplankton samples were taken from the experimental tanks and reservoir and analyzed for total and methylmercury at the experiment start, middle and end (days 0, 14 and 35). These samples were collected following the EPA Method 1631 "clean hands/dirty hands" techniques for mercury tissue sample collection (US EPA 2002). Zooplankton were collected from the reservoir using methods described above, dewatered as much as possible on site, immediately stored in acid-washed glass bottles with Teflon lids, double bagged and flash frozen on dry ice before complete freezing in the lab. Mesocosm zooplankton samples required multiple tows of a Van Dorn sampler and subsequent filtration due to the size limitations of the tanks; sample collection methods were otherwise identical to reservoir methods. Flash frozen samples were shipped on dry ice to the United States Geological Survey for tissue processing and analysis of total and methylmercury. Frozen zooplankton samples were freeze-dried and

homogenized. Samples were then analyzed for total mercury using cold vapor atomic absorption spectroscopy (CVAAS) (EPA Method 245.6) (US EPA 1991). Samples for methylmercury were analyzed using cold vapor atomic fluorescence spectroscopy (CVAFS) (EPA Method 1631) (US EPA 2002). Values were reported as dry weights, and quality assurance protocols including matrix blanks, duplicates and spikes were used.

Water for chlorophyll *a* (chl *a*) analysis was taken weekly using grab samples from the tanks and the reservoir, using 1-L opaque amber HDPE bottles. These water samples were stored on ice in a cooler, then processed on site within hours of collection. Chl *a* concentrations were determined by dividing each water sample into two fractions on site, one of which was filtered through a 35-µm mesh filter, which kept the edible fraction of chl *a* only; the second fraction was unfiltered and used to represent total chl *a*. These divided samples were filtered onto glass fiber filters (1.2-µm pore size) using a hand-held vacuum pump, which were then frozen until analysis in the lab. Filters were soaked in acetone and refrigerated for 20h to extract chl *a*, and concentrations were determined using EPA Method 445 (Arar and Collins 1997), using a TD-7200 fluorometer and a Trilogy Chl *a* NA Module (Turner Designs, Sunnyvale, CA).

Mesocosm and reservoir temperature, dissolved oxygen, and pH were measured weekly. Temperature and dissolved oxygen were recorded using a YSI ProODO (YSI Incorporated, Yellow Springs, OH), and pH was measured using an Extech ExStik II pH meter (Extech Instruments, Nashua, NH). These water quality data were taken at mid-depth of the tanks. Temperature and dissolved oxygen were also measured at 1-m intervals in the reservoir, and pH was measured at the water surface.

Water samples for total nitrogen and total phosphorous were collected on day 0 (after nutrient addition) and on day 35. Nutrients were added once at experiment start to simulate a pulse of nutrient-rich runoff as might occur during a rain event. Water samples for total nitrogen and total phosphorus were taken using grab samples from the tanks and the reservoir using 125-mL HDPE bottles; bottles were put on dry ice shortly after collection and then completely frozen until analysis. On day 0, only the reservoir and the nutrient treatment tanks were sampled (post-nutrient addition) as the nutrient concentrations in the reservoir were representative of the non-nutrient addition tanks at the experiment start. On day 35, all 16 tanks and the reservoir were sampled for nutrient concentrations. Total nitrogen samples were analyzed at the Cooperative Chemical Analytical Laboratory following CCAL 33A.3 method (Cooperative Chemical Analytical Laboratory 2013). Total phosphorous samples were processed using the CCAL 35B.2 method (Cooperative Chemical Analytical Laboratory 2010), and then analyzed using a Shimadzu UV-1800 Spectrophotometer (Shimadzu, Kyoto, Japan).

Statistical analyses

The primary objective of this study was to examine the singular and interactive effects of nutrients and temperature on dependent variables such as mercury concentrations, total and edible chlorophyll *a*, and zooplankton community metrics. Towards that end, two-factor repeated measures analysis of variance (RM-ANOVA), and two-factor ANOVA were run using the EZ package (Lawrence 2013) in R version 3.1.2 (R Development Core Team 2014). Because time can influence response variables, RM-ANOVAs were chosen to account for the lack of independence between sampling dates. This statistical method is commonly used to correct for the influence of time in

observational and longitudinal studies. RM-ANOVAs can also account for the random effects that each individual tank could have on results. Treatments were applied on day 0 and therefore the first week was not included in analyses. Environmental criteria (temperature, dissolved oxygen, and pH) were also compared between treatments using two-factor RM-ANOVA to examine any possible confounding factors. Environmental variables and zooplankton community metric variables from day 0 were tested using a two-factor ANOVA to ensure no statistical differences were present at the start of the experiment, and no significant differences were found. Separate two-way ANOVAs were used to analyze differences in total nitrogen and total phosphorous between treatments on days 0 and 35 (immediately following nutrient addition, and at experiment end). Shapiro-Wilk, Levene's and Mauchly's tests were used to test assumptions of normality, homogeneity and sphericity for the aforementioned analyses. Greenhouse-Geisser corrections (when $\varepsilon<0.75$) were used when the assumption of sphericity was violated.

Exploratory statistical analysis on the dominant taxa (present in >5% of the samples) was done using redundancy analysis (RDA) with the vegan package in R (Oksanen et al. 2015). Zooplankton species abundance data were averaged by treatment for each of the five weeks of the experiment, and transformed with a Hellinger transformation before RDA analysis to correct for high variance in individual species counts (Legendre and Gallagher 2001). The variables of nutrients, temperature, and time were used to explain differences in species abundance, where temperature was continuous and nutrients were categorical. Environmental variable correlations and variance inflation factors showed no evidence of collinearity. Forward selection of the

RDA model was used to find the significant variables affecting species abundance (n=999).

Results

Environmental conditions

The nutrient addition on day 0 effectively raised nutrient levels in nutrient treatment tanks (Figure 5). Total nitrogen (TN) was significantly higher in treatment tanks compared to water from Cottage Grove Reservoir: treatment tanks showed an average 83% increase in TN as compared to the reservoir ($F_{2,6}$=17.47, p=0.003). Total phosphorous (TP) concentrations in the treatment tanks were also significantly different from the reservoir concentrations following the nutrient addition, averaging 35% higher concentrations than the reservoir ($F_{2,6}$=19.36, p<0.001) (Figure 5). These levels of total dissolved nitrogen and phosphorous in the treatment tanks are considered eutrophic, thus achieving the desired treatment (Wetzel 2001).

The second factor of this experiment was to passively warm temperature treatment tanks as compared to control tank water temperatures. There was a significant difference in the temperature treatment: over five weeks, temperature tanks were warmer than controls, averaging 20.6°C (±0.3SE) whereas control tanks averaged 19.9°C (±0.4SE) ($F_{1,12}$=8.38, p=0.001) (Table 1, Figure 6). Reservoir temperatures were consistently warmer than all tanks, though general warming and cooling trends tracked similarly between the tanks and the reservoir (Figure 6).

Edible and total chlorophyll *a* (chl *a*) concentrations were highly variable, but both appeared to spike in the week following the nutrient addition to treatment tanks, and

equilibrated by day 14 (Figure 7). The edible fraction (<35µm) was significantly impacted by time and the interaction of time and nutrients, and showed an average 5% increase over edible chl *a* in nutrient treatment tanks as compared to controls (control average: 0.094 $mg \cdot L^{-1}$, ±0.03SE, nutrient average: 0.10 $mg \cdot L^{-1}$, , ±0.03SE) (Table 1). Treatments did not have a significant effect on total chlorophyll *a*, though there was a weak positive effect of temperature over time (Table 1).

Dissolved oxygen and pH appeared to respond to warming and nutrient treatments, respectively, over the course of the experiment. Dissolved oxygen was significantly impacted by the temperature treatment and the interaction of time and temperature, where control tanks averaged 8.8 $mg \cdot L^{-1}$ (±0.3 SE)over the five weeks of the experiment and temperature treatment tanks averaged 8.2 $mg \cdot L^{-1}$ (±0.2SE) (Appendix A1,A2). Time and the interaction of nutrients and time were the only significant factors impacting pH; however differences were minor (nutrient treatment average= pH 8.64, ±0.15 SE) control average = pH 8.66, ±0.18 SE) (Appendix A1, A2). Over the five weeks of the experiment, all of the tanks trended towards more alkaline pH values and higher dissolved oxygen, which are not uncommon in the later summer months in response to high primary productivity from increased sunlight and warmer temperatures, all conditions which were present in the mesocosms (Wetzel 2001).

Zooplankton methylmercury and total mercury concentrations

Warming and nutrient treatments had a significant interactive effect on the concentrations of methylmercury (MeHg) in the zooplankton: at low temperatures, nutrients had no effect on MeHg, but at high temperatures, the addition of nutrients

reduced MeHg zooplankton concentrations compared to no nutrients (Figure 8, 9, Table 2). Though concentrations in all tanks fell by day 35 as compared to day 14 more striking differences between treatments became apparent, with a significant time × temperature × nutrient interaction (Figure 8, Table 2). Regressions of zooplankton MeHg concentrations as a function of temperature (R^2=0.164, p=0.024) and edible chl *a* (R^2=0.011, p=0.467) both showed negative relationships (Figure 10a,b), though only temperature was a significant predictor; the temperature result however is likely more driven by the sample date (where day 14 was cooler than day 35) than by a correlation between MeHg and temperature.

While the relationship between zooplankton MeHg concentrations and zooplankton and biomass (R^2=0.025, p=0.391) was not significant (Figure 11a,b), there was a weak positive relationship between cladoceran biomass and MeHg concentrations (R^2=0.079, p=0.125), but no relationship between the ratio of cladocerans:copepods and MeHg concentrations (R^2=0.013, p=0.475) (Figure 12). However, zooplankton MeHg concentrations were significantly positively related to abundance-weighted body size (R^2=0.139, p=0.038) (Figure 12f). The MeHg levels in the reservoir zooplankton increased over the experiment, in contrast to the tanks, which showed a decreasing trend, suggesting that demethylation of the methylmercury may have occurred in the tanks over the five weeks of the experiment (Figure 8). No significant effects of treatments on total mercury (THg) concentrations were found at the end of the experiment (Figure 8, Table 2) (due to low zooplankton mass in treatment tanks, THg was only analyzed for day 35).

Zooplankton community and species metrics

Zooplankton community metrics were highly variable, and showed mixed results due to treatments (Figure 13). There was a modestly significant effect of nutrients on zooplankton abundance, with a 33% increase in the nutrient treatment compared to control over the five weeks of the experiment (Figure 13a, Table 3). Biomass showed no significant effects of treatments (Figure 13b, Table 3), though abundance-weighted average body length increased as a result of higher temperature (Figure 13c, Table 3). Regressions showed a significant, positive relationship between edible chl *a* and zooplankton abundance (R^2=0.224, p=0.019) and biomass (R^2=0.169, p=0.046) (Figure 14a,b).

Life history and community composition metrics of the two primary groups of zooplankton, cladocerans and copepods, were measured to see if shifts could potentially explain differences in MeHg concentrations. The ratio of cladoceran:copepod abundances (not including juveniles) was significantly impacted by the interaction of time × nutrients: by experiment end, nutrient treatments were largely dominated by cladocerans (Figure 15, Table 4). However, biomass of cladocerans and copepods were unaffected by treatments (Figure 15, Table 4). Over the course of the experiment, eggs per individual in both cladoceran and copepod groups were significantly impacted by the interaction of nutrients × temperature. Both groups showed declining numbers of eggs per individual in temperature treatments, though nutrients did seem to mitigate temperature effects slightly (Figure 16, Table 4).

The five dominant species of zooplankton found in the tanks and in the reservoir were the cladocerans *Daphnia pulicaria, Bosmina longirostris,* and *Chydorus sphaericus* and the copepods *Mesocyclops edax* and *Skistodiaptomus oregonensis.* The only species that appeared to be impacted by any of the treatment combinations was *D. pulicaria*. The interaction of nutrients × temperature affected abundance of *D. pulicaria* such that abundance decreased in the presence of both stressors relative to the control, but increased with warming and no nutrients (Appendices B1, B2). The results for other species are shown in Appendices B1 and B2. Only *M. edax* showed changes in size over the course of the experiment; average length was significantly increased by nutrients over the course of the experiment (Appendix B1).

A redundancy analysis (RDA) of the abundance of dominant taxa showed changes over time, and relationships between species composition, temperature and nutrients (F=8.407, p=0.001) (Figure 17). RDA axis 1 explained 46% of the variance and was driven largely by temporal changes; RDA axis 2 explained 9% of the variance and was largely defined by temperature and nutrients. The cladoceran *Daphnia pulicaria* was positively correlated with time and nutrients, while *Chydorus sphaericus* (cladoceran) showed a relationship with time and temperature (Figure 17). The copepods *Skistodiaptomus oregonensis* and *Mesocyclops edax* were most abundant on weeks 1 and 2, and show a negative relationship with time, corresponding with data showing that tanks became dominated by cladocerans as the experiment progressed. Nutrients influenced species composition primarily on weeks 2 and 3, corresponding with a chl *a* spike at the end of week 1 (Figure 7). The influence of temperature on species

composition was greatest in weeks 5 and 6 when some of the warmest temperatures were recorded (Figure 6).

Discussion

The interactions of contaminants with other anthropogenic stressors, like climate change and excess nutrients, in freshwater systems have the potential to compound the effects of those pollutants. Therefore, understanding how contaminants might interact with these stressors becomes more critical in order to predict how systems may respond in the future. The primary aim of this project was to determine if the effects of warmer temperatures and excess nutrients would alter zooplankton communities and phytoplankton biomass, and thus in turn impact MeHg concentrations in zooplankton (Figure 1). The key findings from this study are, first and foremost, that nutrients mediate the effect of temperature on MeHg concentrations in zooplankton (Figure 9). Further, I found that temperature had little, if weak effects on phytoplankton and zooplankton biomass, but did appear to shift community composition towards larger-bodied species. Last, nutrients did increase phytoplankton as was expected, and further, seemed to support an increased ratio in the abundance of cladocerans to copepods. These results answer some questions about the relationships between plankton and resulting MeHg concentrations, but raise others as to the precise mechanisms that could be changing contaminant concentrations (Figure 17).

The finding that an increase in nutrients appears to buffer zooplankton MeHg concentrations in the presence of warmer temperatures supports my hypothesis that a nutrient-driven increase in phytoplankton would mitigate increased zooplankton metal concentrations precipitated by higher temperatures (Figure 9). This result is supported by other research where systems with higher phytoplankton concentrations appeared to have lower fish tissue MeHg concentrations (Pickhardt et al. 2002, Chen and Folt 2005, Chen

et al. 2005). However, unlike many MeHg studies, my research is fairly unique in that the focus is on zooplankton, the dietary source of mercury bioaccumulation for many fish in freshwater systems (Morel et al. 1998, Boening 2000). Though the nutrient treatment in this experiment consisted of a single pulse of added nutrients, the effect was significant enough to elevate edible chlorophyll *a* concentrations (Figure 7). This increase in edible phytoplankton may have diluted the existing concentrations of MeHg present in the nutrient treatment, thus resulting in lower MeHg zooplankton concentrations. While a regression of both MeHg sampling dates and chl *a* found no relationship (Figure 10b), a regression of day 14 data alone (shortly after the phytoplankton responded to nutrient pulse) found that MeHg concentrations had a weak negative correlation with increasing chl *a* (R^2=0.089, p=0.279). An alternative explanation is that biodilution via increased zooplankton density could explain differences in MeHg concentrations (Chen and Folt 2005). However, this seems unlikely as total zooplankton abundance and biomass were not significantly increased by any treatments (Figure 12). It is important to note that although nutrients appear to have mitigated the effect of temperature on zooplankton MeHg concentrations, nutrient tanks still had higher median MeHg values than controls, which does not support my initial hypothesis that nutrient tanks would have the lowest MeHg concentrations. An increase in organic material in the nutrient tanks, caused by phytoplankton die-off, could account for this pattern of elevated MeHg in just the nutrient tanks, as studies have found that in some systems, such as wetlands, increased carbonaceous material can serve as medium for the bacterial methylation of mercury (Zillioux et al. 1993).

Another central finding of this experiment was that, contrary to expectation, temperature did not significantly increase the biomass of zooplankton or phytoplankton (though there was a weakly positive effect on total chl *a*). However, higher temperatures did shift community composition to larger-bodied zooplankton species based on abundance-weighted body size (Figure 13), partially supporting my prediction that warmer temperatures would benefit larger-bodied species. Also, while temperature had no effect on individual species length, temperature did decrease the number of eggs per individual in both groups, indicating that a metabolic trade-off may have occurred, where maintaining body size was a greater priority than reproduction under stressful, warmer conditions (Appendix B2, Table 3; Orcutt and Porter 1984, Weetman and Atkinson 2004). Further, larger-bodied, less selective grazers, such as daphniid cladocerans, can fare better in warmer systems, as these filter-feeding species are more generalist feeders (Brett et al. 2000, Sommer and Stibor 2002). Cladocerans also have higher metabolic rates than copepods (Sommer and Stibor 2002) and it is well established that warmer temperatures can result in higher filtering rates, especially in *Daphnia* (Burns 1969). Thus, two lines of evidence, i.e., the increase in larger-bodied grazers and a weak increase in phytoplankton, suggest that increased grazing pressure may have dampened the response of phytoplankton to warmer temperatures. It is also possible that the predicted increase in chl *a* concentrations resulting from increased temperature did not occur because the temperature increase was too minor to illicit a response in phytoplankton (McKee et al. 2003), but may have positively impacted periphyton growing on insides of tanks. Anecdotally, periphyton growth over the course of the experiment was such that by day 35, the insides of tanks were fairly uniformly coated

with periphyton, thereby possibly limiting detectable increases in chl *a* as phytoplankton. The response of plankton communities to temperature is nuanced and likely subject to trophic dynamics that are more subtle than could be tested within the scope of this project.

Another important finding from this study was that, as expected, nutrients increased edible phytoplankton, although the effect changed over time. However, nutrients also increased the ratio of cladocerans:copepods, which I did not predict. These results are interesting because excess nutrients, most often due to agricultural run-off, have been associated with shifts to lower food quality species of algae, including cyanobacteria (Sommer and Stibor 2002, Heisler et al. 2008, Zhao et al. 2008). The unexpected increase in abundance in the cladoceran:copepod ratio could be attributed to the generalist feeding pattern of cladocerans, where food is not selected but rather filtered (Sommer and Stibor 2002). It is possible that nutrients created lower quality phytoplankton communities but that the cladocerans were able to use it more effectively than the more selective copepods (Sommer and Stibor 2002). Regardless of the mechanism, the increase in cladoceran:copepod abundance is clear by experiment end: the cladocerans *Daphnia pulicaria* and *Chydorus sphaericus* dominate (Figure 17).

The central results of this experiment point to a general trend that changes in both the phytoplankton and zooplankton community were induced by temperature and nutrients, to varying degrees; but did these changes in plankton metrics ultimately impact zooplankton MeHg concentrations? My initial prediction that increased zooplankton biomass would reduce zooplankton MeHg was not supported, and the impact that phytoplankton had on reducing MeHg concentrations was weaker than I anticipated.

However, increased *Daphnia pulicaria* abundance (R^2=0.109, p=0.069), biomass (R^2=0.086, p=0.109) and abundance-weighted body size (R^2=0.139, p=0.038) were correlated with increased MeHg concentrations, which while not precisely predicted, correspond with existing literature on the positive relationships between larger body size and increased MeHg concentrations (Kainz et al. 2006). While some of the mechanisms I expected to impact MeHg concentrations were not apparent, there were indications that, with further and finer-grained examination of species-specific metabolic rates and affinity or defense against metal bioaccumulation, underlying trophic interactions could explain these differences in MeHg.

Phytoplankton taxonomic identification was beyond the scope of this project; however, species-level differences in algae could possibly account for some of the variance in zooplankton MeHg concentrations. Some species of algae like *Chlorella* have been found to be "hyper-accumulaters" of heavy metals, and still others, like *Anabaena,* produce extra-cellular compounds that appear to act as a defense against metal uptake (Reed and Gadd 1989). Further, low light conditions seem to limit algal uptake of metals in several species (Reed and Gadd 1989). Based on phytoplankton community assemblages, and potentially differing light levels in treatment tanks due to shade, MeHg uptake by zooplankton could then vary extensively based on what phytoplankton species are present and in what light conditions they were feeding.

Similarly, zooplankton community assemblages and species-specific metabolic function could have a more nuanced influence on MeHg concentrations than was initially predicted. Based on a combination of factors, from feeding preferences (Sommer and Stibor 2002) to percentage of an organism's essential fatty acids (Kainz et al. 2008),

cladocerans generally take up MeHg more efficiently than copepods in the same systems (Pickhardt et al. 2005, Stewart et al. 2008). Given this information, one would expect the relationship between cladocerans and MeHg to be clear-cut; however, the results are not so definitive. There was a weak positive relationship between cladoceran biomass and MeHg concentrations (R^2=0.079, p=0.125), but no relationship between the ratio of cladocerans:copepods and MeHg concentrations (R^2=0.013, p=0.475). However, as mentioned previously zooplankton MeHg concentrations were significantly positively related to abundance-weighted body size and weakly positively related to *Daphnia pulicaria* abundance and biomass. This result indicates that large-bodied species and some cladocerans are perhaps more efficient than others at bioaccumulation of metal, based on feeding preferences, temperature sensitivity and metabolic rates (DeMott 1982).

As with any mescocosm experiment, confounding variables have the potential to detract from the results if not adequately accounted for in the initial experimental design. Given that the experiment ran for five weeks, time was a factor in the results. I chose to use the repeated measures ANOVA (RM-ANOVA), a robust statistical method that corrects for lack of independence between data points (e.g., zooplankton abundance on day 14 is dependent upon zooplankton abundance on day 7). It is possible that changes in any of the metrics I measured, from zooplankton abundance to methylmercury concentrations, could be due solely to time; however, the RM-ANOVA partitions the variability potentially caused by the time factor, and therefore any significant results from this study that were solely attributed to time were not considered treatment effects, and as such, discounted. In cases where time and another treatment interacted significantly, it

meant that the treatment had an effect that changed over time (e.g., edible chlorophyll *a*, Table 1).

Other potential time effects to mesocosms include increases in pH, dissolved oxygen and periphyton growth over the five week experiment. These water quality variables are expected to shift over time, especially given the season of the experiment: increased solar radiation and warmer air temperatures in summer months tend to result in higher primary productivity, which can increase pH values and produce systems with higher dissolved oxygen (Wetzel 2001). Periphyton on the insides of tanks was qualitatively observed to increase by experiment end, and this time effect may or may not have had some bearing on the lack of temperature effect to chlorophyll *a* as phytoplankton; however, periphyton chl *a* values were not measured as periphyton is not part of the zooplankton diet crucial to MeHg uptake.

Another factor that may have played a role in results is the process of photodemethylation, where methylmercury in the water column and in organisms is converted back to elemental Hg through UV radiation; the elemental Hg then volatilizes out of the system (Lehnherr and St. Louis 2009). Other pathways, including bacterial demethylation, could have also contributed to MeHg losses (Seller et al. 1996, Marvin-Dipasquale et al. 2000). These processes could explain the drop in methylmercury across all treatment combinations from day 14 to day 35 (Figure 8). Given that I did not use sediments in my mesocosms, which act as MeHg producers in contaminated systems like Cottage Grove Reservoir, there was no opportunity for the Hg to cycle back into MeHg in the tanks. Further, the interactive temperature and nutrient effect I saw could have only impacted existing zooplankton MeHg concentrations, as without sediments the

regeneration of MeHg in mesocosms was likely near zero. This fact, in concert with the likely loss of MeHg to photodemethyation, limited the existing MeHg in mesocosms over time. Because of this reduction, it is possible that potential treatment effects were minimized or overshadowed by overall loss of MeHg in these simulated systems, thus my results are likely conservative (Figure 9).

Even with these diminished MeHg concentrations, I was able to observe an interactive effect of temperature and nutrients on MeHg concentrations. This finding adds to the current understanding of why mercury concentrations might fluctuate in differing conditions of both primary productivity and temperature, both factors which regularly affect reservoirs in particular, but on a larger scale, also impact what are typically considered more pristine environments like Arctic ecosystems (Stern et al. 2012). Clearly the connection between zooplankton MeHg concentrations and fish tissue concentrations is significant: without a precise point source of mercury, 2003 samples of the mainstem of the Willamette River in Oregon showed concentrations of 0.47 $mg \cdot kg^{-1}$ in fish tissue, while fish tissue concentrations in Cottage Grove Reservoir, upstream of the mainstem, averaged 1.63 $mg \cdot kg^{-1}$ (Hope and Rubin 2005). Cottage Grove Reservoir zooplankton averaged 0.14 $mg \cdot kg^{-1}$ MeHg in 2013 (this study). While these are averages, and sample dates differ by ten years, these values represent a theoretical increase of over 1,000%, or three orders of magnitude from zooplankton MeHg to fish MeHg in Cottage Grove Reservoir. Clearly, zooplankton mercury concentrations have a significant impact on the MeHg in fish, and ultimately, the MeHg that could be consumed by humans. As long as coal combustion persists, the legacy of mercury contamination in both marine and freshwater systems will continue. Gaining a better understanding of what might mitigate

or amplify its harmful effects is critical to present and future generations of people reliant on fisheries for recreation and consumption.

Chapter 3: Conclusion

The state of Oregon is home to some of the most actively mined mercury sources in the United States, and is therefore no stranger to the adverse effects of this pollutant in its waterbodies (Gray 2013, ODEQ 2016). In fact, fish consumption advisories due to mercury have been in effect at Cottage Grove Reservoir since 1979, which was a primary reason for using this site for my experiment, and the basis for my inquiry as to possible factors affecting mercury bioaccumulation in this particular food web (Newell et al. 1996). At present, there are 37 waterbodies in Oregon that are 303d listed for having exceeded the state water quality standard for mercury in fish tissue, as mandated by the Oregon Department of Environmental Quality (ODEQ 2006, ODEQ 2016). Another 14 waterbodies are listed as having potential concern over Hg concentrations in samples, and waterbodies are very infrequently de-listed for mercury, unlike other pollutants like bacteria (ODEQ 2016). While the beneficial uses covered by the Clean Water Act include protecting waterbodies for recreational use, mercury is particularly concerning because of its threats to more critical beneficial uses, such as aquatic life, drinking water and human health (U.S. Congress 1972).

Mercury's effects extend well beyond the state of Oregon: it is listed as one of the top three sources of water quality impairment in evaluated U.S. lakes (the Great Lakes in particular), wetlands, coastal shorelines, and near coastal and ocean waters, affecting waterbodies in 48 states (U.S. EPA 2009, Kubasek and Silverman 2011). Worldwide, atmospheric movement of mercury through particulate emissions from coal combustion, chlor-alkali plants, deforestation and smaller, natural sources like volcanic eruptions deposit mercury in aquatic systems that are thousands of miles from any mercury point

source (Hammerschidt and Fitzgerald 2006). While coal combustion as an energy source is decreasing in developed countries, developing nations are still burning coal at exponential rates, and most often using less advanced technology to "clean" the resulting emissions than is used in the United States (Jaffe et al. 2005, Driscoll et al. 2013). The scrubbers frequently used in more modern coal combustion plants still only reduce the amount of mercury in emissions by 37% (Gray 2003). Further, mercury is a persistent pollutant, and it has been calculated that even if all anthropogenic sources of mercury were to cease, it would still remain in global aquatic environments for 20 years to come, as a conservative estimate (Mason et al. 1994). Even in my short term experiment, with no additional sources of mercury during the five week project, MeHg still maintained consistently high levels in zooplankton, a key factor in bioaccumulation. As anthropogenic inputs of mercury show no signs of ceasing, gaining a better understanding of the mechanisms behind bioaccumulation of this neurotoxin in aquatic systems seems critical.

Mercury deposition from coal combustion is only one by-product of the Industrial Age that has negative consequences for aquatic systems. The advent of fertilizers, paired with booming human populations that rely on agriculture to sustain this new growth has resulted in exponential increases in fertilizer use, and corresponding unprecedented volumes of nutrient-rich runoff finding its way into streams, rivers, lakes and oceans (Ramankutty and Foley 1999, Smith and Schindler 2009). In my experiment I found that even with a single pulse of high concentrations of nitrogen and phosphorous, chlorophyll concentrations increased and had consequential impacts to zooplankton abundance, biomass and composition. Based on current human population growth models, enough

food can only be produced through better food distribution, increased agricultural yield, and the increased conversion of natural areas to croplands (Young 1999, Ramunkutty et al. 2002). This converted cropland will require more fertilizer, and thus the cycle of continued nutrient runoff and consequential eutrophication of waterbodies due to agriculture will increase as global development continues (Smith et al. 1999). The snapshot of eutrophication effects that I saw in my study is conservative when you consider the scale and magnitude of the global impacts this stressor has to aquatic systems.

A third aquatic stressor that correlates with global development is climate change. Since the Industrial Revolution, global average temperatures have increased by 0.8°C since the mid-1800s (Hartmann et al. 2013). Aquatic food web shifts due to warmer temperatures are already underway, with adverse effects to colder-water species (Isaak et al. 2010), temporal mismatches between zooplankton and their food sources (Winder and Schindler 2004), and a loss of aquatic species diversity due to the loss of temperature-sensitive species and viable habitat (Ficke et al. 2007). The very modest increase of only 0.5°C in my experiment resulted in a zooplankton community shift that favored larger grazers over smaller, more selective copepods. Given that long term climate models predict, conservatively, that global average temperatures may increase by 3°C by year 2080, increasingly warming waters are inevitable, and aquatic ecosystems will either adapt or perish (Mote and Salathe 2010, Hartmann et al. 2013).

Active management of Hg-contaminated freshwater systems could benefit from a better understanding of methylation processes, and the impacts that warming and excess nutrients may have on MeHg production and bioaccumulation in reservoirs in particular.

Several studies have noted the adverse impacts of the "reservoir effect" where the filling and emptying of a flood-management reservoir ultimately contributes more MeHg to the system because of the exposure and consequential re-wetting of sediments (Kuwabara et al. 2005, Stewart et al. 2008, Eckley et al. 2015). This drying and re-wetting stimulates mercury methylation by sulfate- and iron-reducing bacteria (Kuwabara et al. 2005, Stewart et al. 2008, Eckley et al. 2015). Though many reservoirs are used recreationally, seasonal drawdown based on boater and recreational use may unintentionally be contributing to increased MeHg concentrations in these systems.

In some cases, such as Fall Creek Reservoir in Oregon, reservoir drawdown proves to be beneficial for some species (USACE 2014), yet may have unintended effects to other processes, like methylmercury production in sediments. Bull trout (*Salvelinus confluentus*) have shown greater survival rates with a dramatic drawdown of Fall Creek Reservoir (USACE 2014), but shallower and likely warmer waters can result in higher mercury methylation in these exposed sediments, especially during the warmer summer months. Because bull trout are considered threatened under the Endangered Species Act, prioritizing recovery of their populations is essential, and yet consideration of unintended impacts to other environmental concerns, such as increased potential for MeHg bioaccumulation, should be considered as well.

Other projects, such as the water temperature control tower at Cougar Dam in Oregon, may have the opposite effect, where a plan developed to protect a threatened species may simultaneously reduce the potential for increased MeHg production downstream of a reservoir. The construction of a temperature control facility at Cougar Dam in Oregon will ideally improve temperature conditions for bull trout in the

McKenzie River (USACE 2006), and perhaps as a by-product, decrease the potential for late-summer mercury methylation in sediments, as the cooler water released by the new facility will mean lower temperatures downstream of the reservoir, where high levels of MeHg can occur; Eckley et al. (2015) found some of the highest water MeHg concentrations in reservoir outflows in late summer, as opposed to the reservoir water itselt. Downstream flushing of mercury-contaminated water from a reservoir has also been considered as a MeHg mitigation option, though this method can prove to be cost-prohibitive and potentially at odds with other management concerns (Mailman et al. 2006). Consideration of how fisheries and reservoir management might impact mercury methylation and subsequent bioaccumulation can, as may prove to be the case with Cougar Reservoir, be mutually beneficial.

The confluence of climate change, increasing eutrophication of waterbodies and the continued atmospheric deposition of persistent pollutants like mercury confound the problems each of these stressors causes individually. It creates a scenario where better understanding the interactions of these stressors becomes critical to protecting human health (Booth and Zeller 2005, Moss 2011). Global food demand and decrease in arable land due to climate change means fisheries become more and more important for supporting global food needs (Ramunkutty et al. 2002, Ficke et al. 2007). Learning what mechanisms might protect the health of those fisheries from the onslaught of continued pollutants can help sustain critical food sources for future generations.

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Table 1. Statistical summary of RM-ANOVA on water quality data of temperature, and chl *a* edible and total concentrations. Subscripts indicate degrees of freedom for RM-ANOVA. † $p<0.1$; * $p<0.05$.

Variable	Treatment	F ratio	*p* Value
Temperature	$Nutrient_{[1,12]}$	0.005	0.946
	$Temp_{[1,12]}$	8.381	0.001*
	Nutrient x $Temp_{[1,12]}$	4.633	0.509
	$Time_{[4,48]}$	21.336	<0.001*
	Time x $Nutrient_{[4,48]}$	0.543	0.705
	Time x $Temp_{[4,48]}$	6.124	<0.001*
	Time x Nutrient x $Temp_{[4,48]}$	0.229	0.921
Chl *a*, edible	$Nutrient_{[1,12]}$	0.001	0.972
	$Temp_{[1,12]}$	0.181	0.678
	Nutrient x $Temp_{[1,12]}$	0.031	0.862
	$Time_{[4,48]}$	5.842	<0.001*
	Time x $Nutrient_{[4,48]}$	3.627	0.012*
	Time x $Temp_{[4,48]}$	1.223	0.313
	Time x Nutrient x $Temp_{[4,48]}$	0.656	0.626
Chl *a*, total	$Nutrient_{[1,12]}$	0.023	0.881
	$Temp_{[1,12]}$	0.137	0.718
	Nutrient x $Temp_{[1,12]}$	0.031	0.994
	$Time_{[4,48]}$	16.672	<0.001*
	Time x $Nutrient_{[4,48]}$	<0.001	0.103†
	Time x $Temp_{[4,48]}$	2.305	0.072†
	Time x Nutrient x $Temp_{[4,48]}$	1.204	0.322

Table 2. Statistical summary of RM-ANOVA on zooplankton methylmercury concentrations combining mid and endpoint data, and two-way ANOVA statistics on total mercury at experiment end. Subscripts indicate degrees of freedom for RM-ANOVA. † $p<0.1$; * $p<0.05$.

Variable	Treatment	F ratio	*p* Value
MeHg in zooplankton	$Nutrient_{[1,12]}$	0.022	0.886
	$Temp_{[1,12]}$	0.714	0.415
	Nutrient x $Temp_{[1,12]}$	6.423	0.026*
	$Time_{[5,60]}$	57.923	<0.001*
	Time x $Nutrient_{[5,60]}$	2.803	0.085
	Time x $Temp_{[5,60]}$	0.525	0.587
	Time x Nutrient x $Temp_{[5,60]}$	6.603	0.006*
Total Hg in zooplankton	$Nutrient_{[1,12]}$	0.613	0.449
	$Temp_{[1,12]}$	0.049	0.828
	Nutrient x $Temp_{[1,12]}$	1.073	0.321

Table 3. Statistical summary of RM-ANOVA on zooplankton community data of abundance, biomass, total eggs and eggs per individual and abundance-weighted body size for weeks 2 – 5. Subscripts indicate degrees of freedom for RM-ANOVA.
† $p<0.1$; * $p<0.05$.

Variable	Treatment	F Ratio	*p* Value
Abundance	$Nutrient_{[1,12]}$	4.566	0.054†
	$Temp_{[1,12]}$	4.157	0.064†
	Nutrient x $Temp_{[1,12]}$	0.251	0.626
	$Time_{[4,48]}$	0.882	0.482
	Time x $Nutrient_{[4,48]}$	1.493	0.219
	Time x $Temp_{[4,48]}$	0.299	0.877
	Time x Nutrient x $Temp_{[4,48]}$	1.586	0.193
Biomass	$Nutrient_{[1,12]}$	1.066	0.322
	$Temp_{[1,12]}$	2.273	0.158
	Nutrient x $Temp_{[1,12]}$	0.006	0.225
	$Time_{[4,48]}$	0.199	0.902
	Time x $Nutrient_{[4,48]}$	1.475	0.236
	Time x $Temp_{[4,48]}$	0.721	0.551
	Time x Nutrient x $Temp_{[4,48]}$	0.435	0.736
Abundance-weighted average body size	$Nutrient_{[1,12]}$	0.091	0.768
	$Temp_{[1,12]}$	4.978	0.045*
	Nutrient x $Temp_{[1,12]}$	0.799	0.389
	$Time_{[4,48]}$	0.400	0.758
	Time x $Nutrient_{[4,48]}$	0.071	0.977
	Time x $Temp_{[4,48]}$	0.170	0.919
	Time x Nutrient x $Temp_{[4,48]}$	0.785	0.513

Table 4. Statistical summary of RM-ANOVA on cladoceran and copepod community metrics for weeks 2 – 5. Subscripts indicate degrees of freedom for RM-ANOVA. † $p<0.1$; * $p<0.05$.

Variable Treatment	F ratio	*p* Value	Variable Treatment	F ratio	*p* Value
Cladoceran eggs per individual			**Copepod eggs per individual**		
$Nutrient_{[1,12]}$	0.551	0.472	$Nutrient_{[1,12]}$	2.251	0.159
$Temp_{[1,12]}$	2.841	0.177	$Temp_{[1,12]}$	0.162	0.694
Nutrient x $Temp_{[1,12]}$	4.768	0.046*	Nutrient x $Temp_{[1,12]}$	5.390	0.039*
$Time_{[4,48]}$	14.517	<0.001*	$Time_{[4,48]}$	4.155	0.011*
Time x $Nutrient_{[4,48]}$	0.919	0.398	Time x $Nutrient_{[4,48]}$	0.634	0.605
Time x $Temp_{[4,48]}$	0.877	0.413	Time x $Temp_{[4,48]}$	0.211	0.904
Time x Nutrient x $Temp_{[4,48]}$	0.763	0.456	Time x Nutrient x $Temp_{[4,48]}$	0.427	0.744
Cladoceran abundance			**Copepod abundance**		
$Nutrient_{[1,12]}$	0.193	0.668	$Nutrient_{[1,12]}$	0.089	0.769
$Temp_{[1,12]}$	0.028	0.871	$Temp_{[1,12]}$	0.102	0.755
Nutrient x $Temp_{[1,12]}$	3.037	0.107	Nutrient x $Temp_{[1,12]}$	0.222	0.646
$Time_{[4,48]}$	2.899	0.086†	$Time_{[4,48]}$	0.183	0.946
Time x $Nutrient_{[4,48]}$	1.369	0.273	Time x $Nutrient_{[4,48]}$	1.148	0.346
Time x $Temp_{[4,48]}$	0.377	0.654	Time x $Temp_{[4,48]}$	1.746	0.155
Time x Nutrient x $Temp_{[4,48]}$	0.345	0.676	Time x Nutrient x $Temp_{[4,48]}$	0.915	0.463
Cladoceran biomass			**Copepod biomass**		
$Nutrient_{[1,12]}$	0.304	0.592	$Nutrient_{[1,12]}$	0.053	0.822
$Temp_{[1,12]}$	0.074	0.789	$Temp_{[1,12]}$	0.794	0.391
Nutrient x $Temp_{[1,12]}$	0.265	0.616	Nutrient x $Temp_{[1,12]}$	0.053	0.821
$Time_{[4,48]}$	0.454	0.769	$Time_{[4,48]}$	1.554	0.202
Time x $Nutrient_{[4,48]}$	0.733	0.574	Time x $Nutrient_{[4,48]}$	0.501	0.735
Time x $Temp_{[4,48]}$	0.588	0.673	Time x $Temp_{[4,48]}$	0.095	0.984
Time x Nutrient x $Temp_{[4,48]}$	1.009	0.412	Time x Nutrient x $Temp_{[4,48]}$	0.815	0.522
Cladoceran:copepod abundance ratio					
$Nutrient_{[1,12]}$	0.146	0.709			
$Temp_{[1,12]}$	0.812	0.385			
Nutrient x $Temp_{[1,12]}$	2.820	0.119			
$Time_{[4,48]}$	6.257	<0.001*			
Time x $Nutrient_{[4,48]}$	3.126	0.032*			
Time x $Temp_{[4,48]}$	1.142	0.347			
Time x Nutrient x $Temp_{[4,48]}$	1.194	0.326			

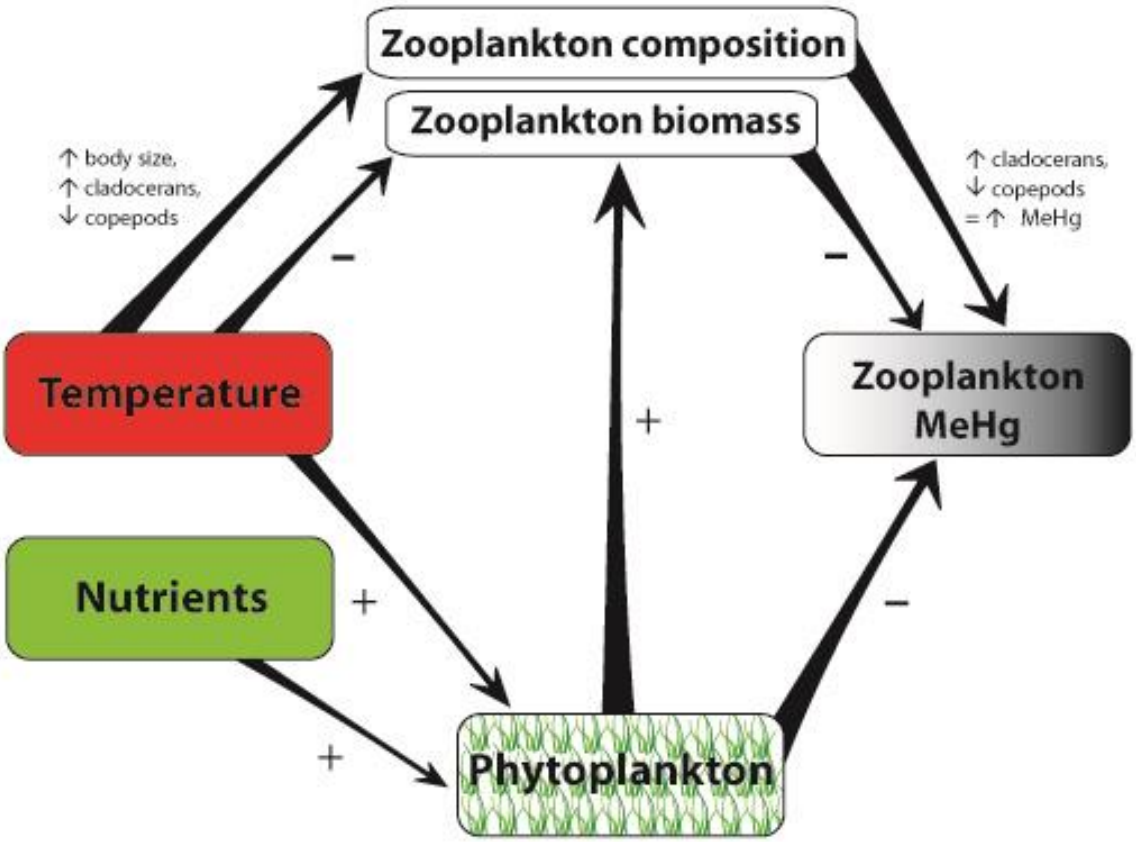


Figure 1. Conceptual model of predicted relationships between stressors (temperature and nutrients) and zooplankton, phytoplankton and zooplankton methylmercury concentrations in a freshwater system. Plus signs represent a predicted increase; minus signs represent a predicted decrease.

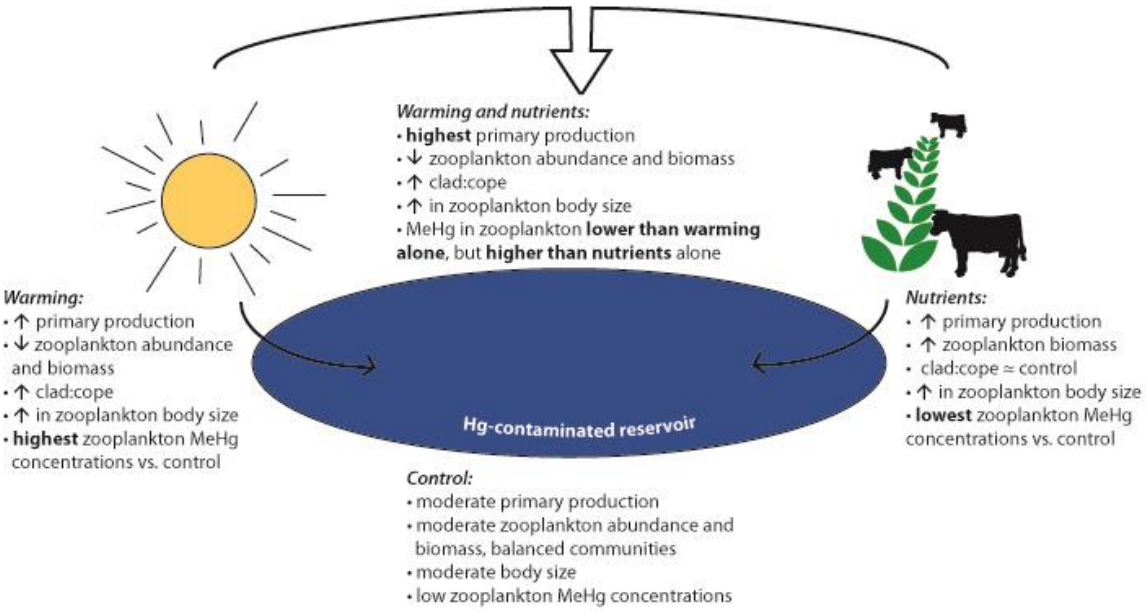


Figure 2. Conceptual model of predicted results of treatment combinations using mercury-contaminated zooplankton from Cottage Grove Reservoir, Oregon. Clad:cope = abundance ratio of cladocerans to copepods.

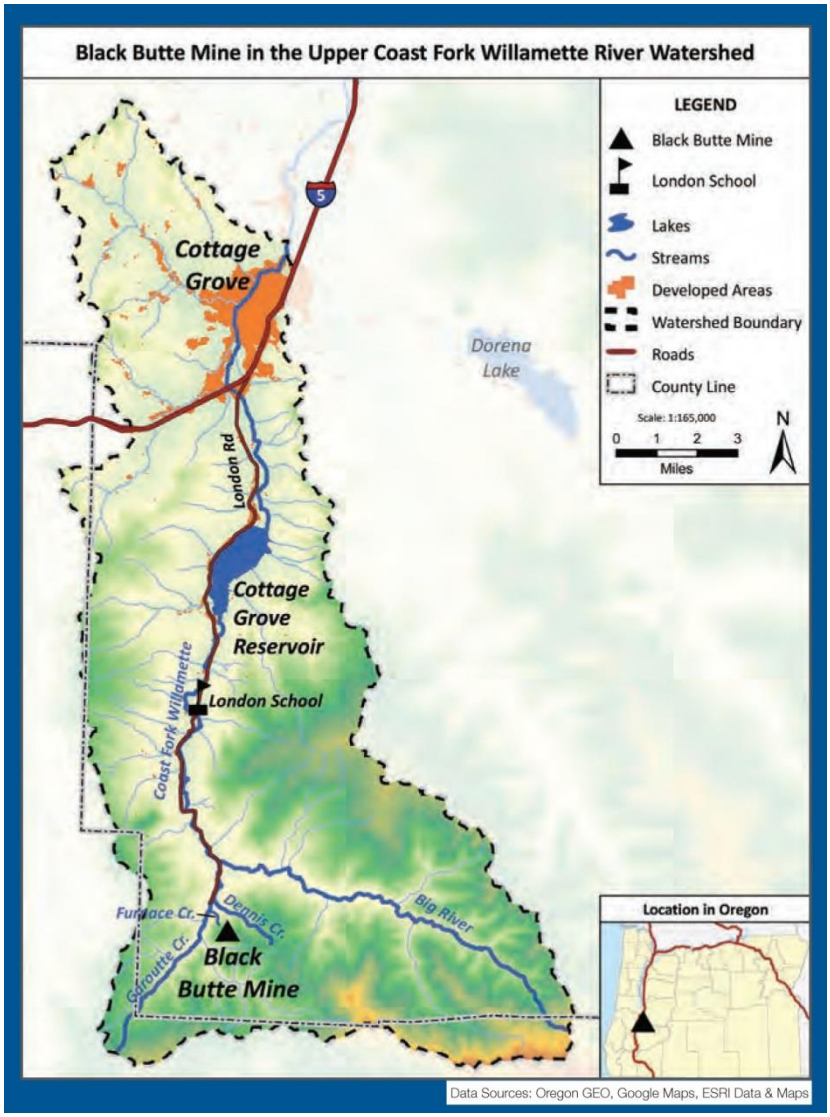


Figure 3. Map of Cottage Grove Reservoir and its proximity to the Black Butte Mine site, reprinted courtesy of the Oregon Health Authority, 2013.

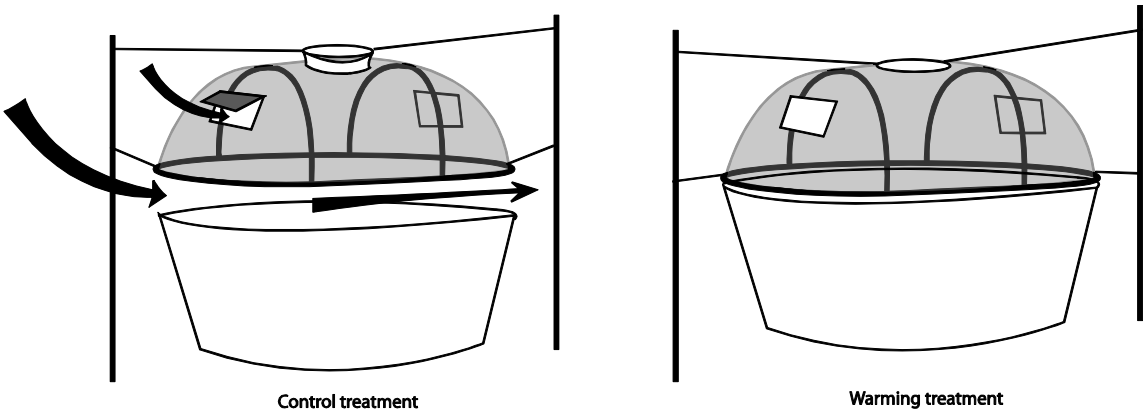


Figure 4. Schematic of greenhouse canopies used for passive warming treatment, as adapted from design in Strecker et al. (2004). When canopy is raised and vents opened, it is a control. When canopy is lowered onto the edge of tank and vents are closed, it is a warming treatment, intended to raise water temperatures by approximately 0.5˚C.

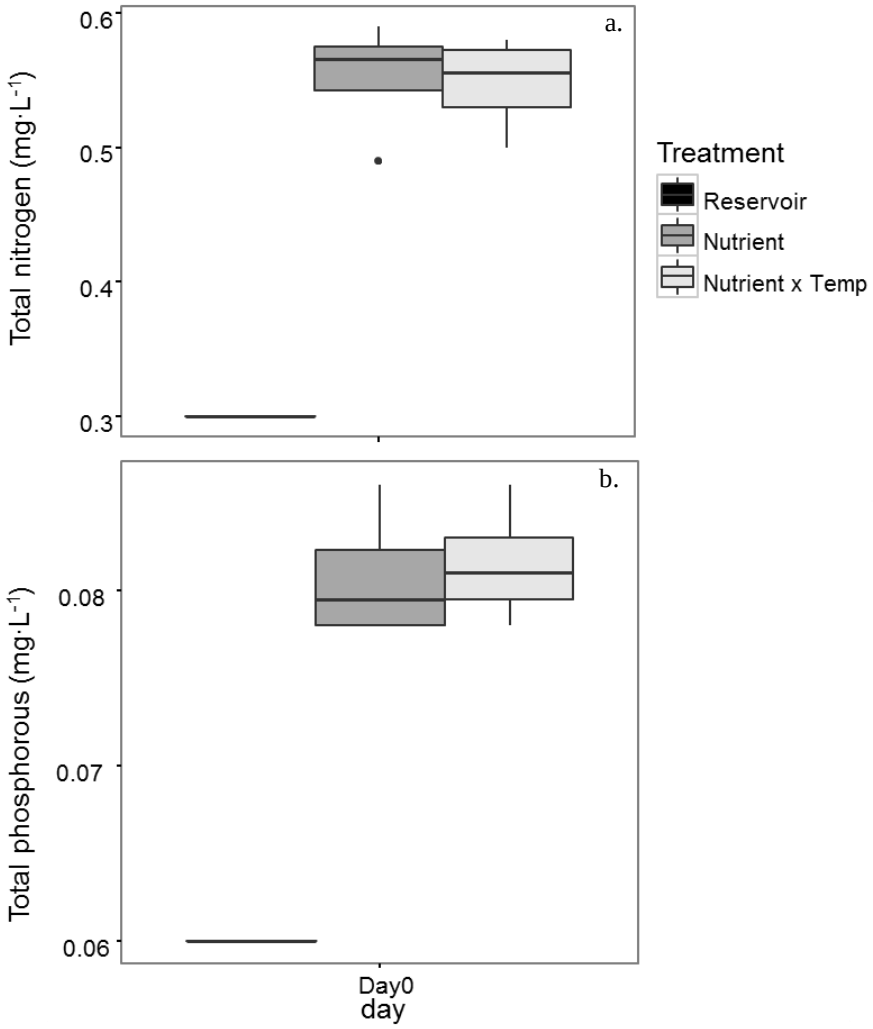


Figure 5. (a) Total nitrogen and (b) total phosphorous water concentrations ($mg \cdot L^{-1}$) by treatment combination after nitrogen addition, as compared to reservoir. Box represents interquartile range of values, with horizontal line as the median; whiskers represent minimum and maximum values. Reservoir nutrient concentrations are representative of control tanks on Day 0.

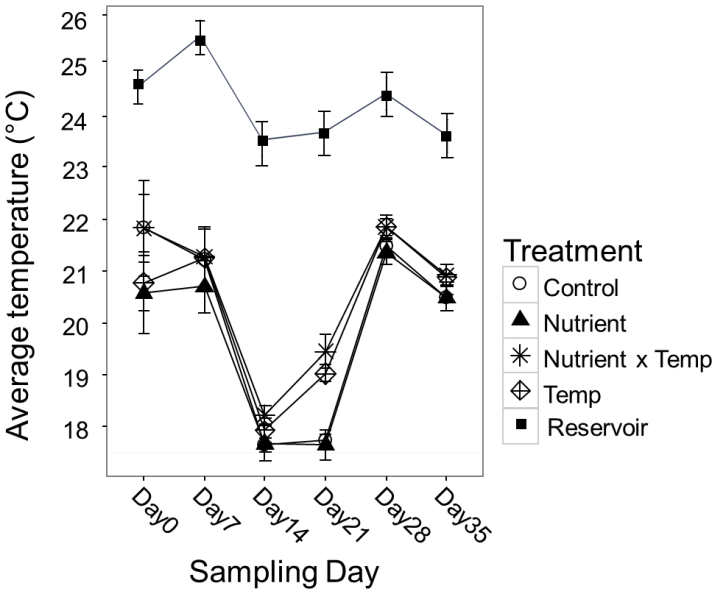


Figure 6. Average water temperature by treatment combination by week (˚C). Error bars represent ±1 SE.

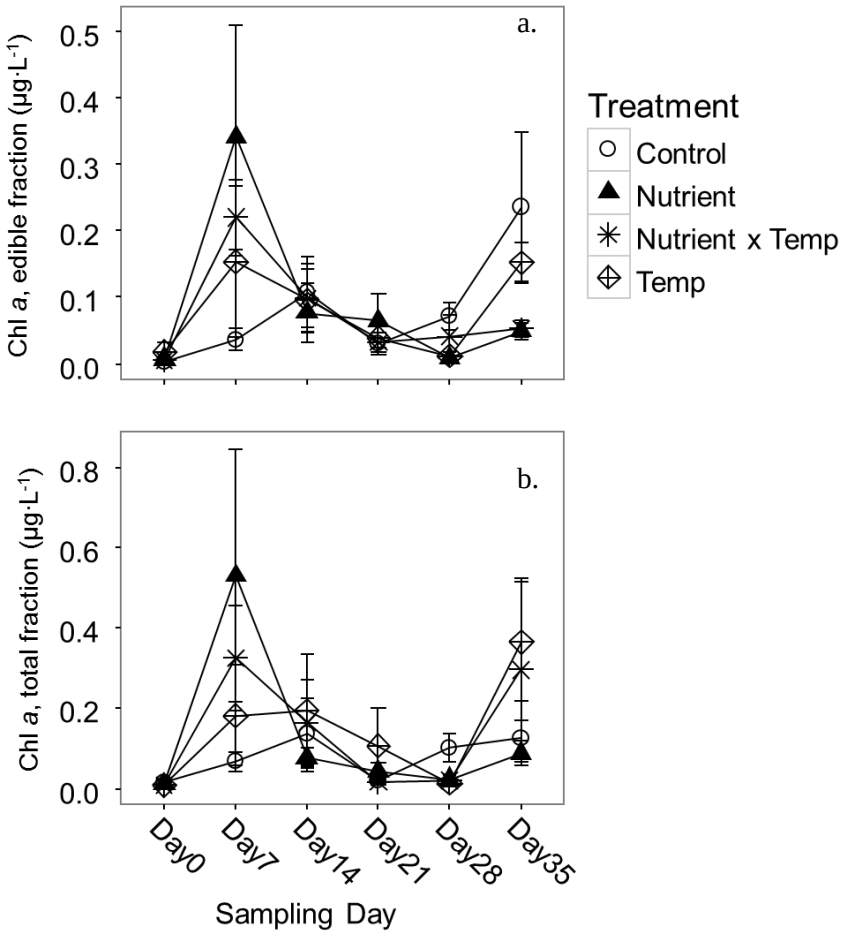


Figure 7. (a) Edible and (b) total chlorophyll *a* averages ($\mu g \cdot L^{-1}$) by treatment combination by week. Error bars represent ±1 SE.

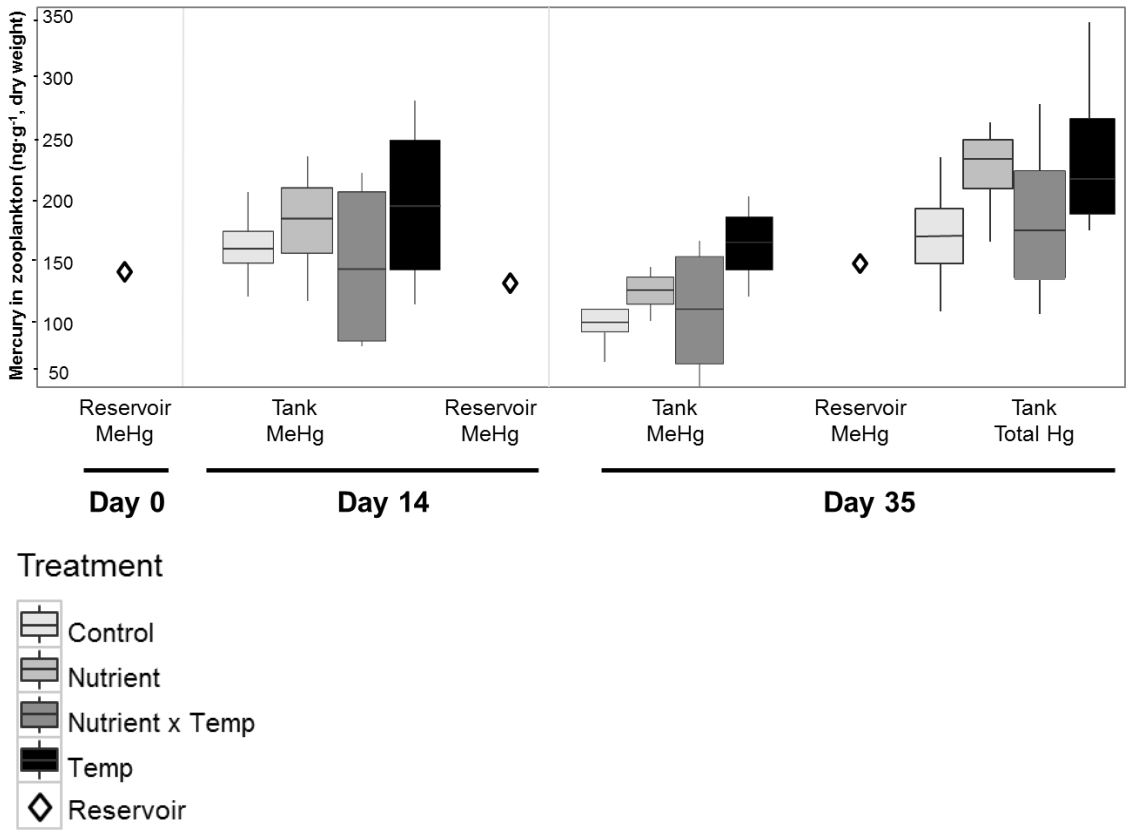


Figure 8. Box and whisker plots of methylmercury and total mercury in zooplankton ($ng \cdot g^{-1}$, dry weight). Box represents interquartile range of values, with horizontal line as the median; whiskers represent minimum and maximum values. Single values represent single measurements from the reservoir zooplankton; on day 0, reservoir zooplankton MeHg values are considered representative of values in tank zooplankton. Methylmercury in tank zooplankton was measured on day 14 and on day 35 of the experiment, and total mercury was only measured on day 35.

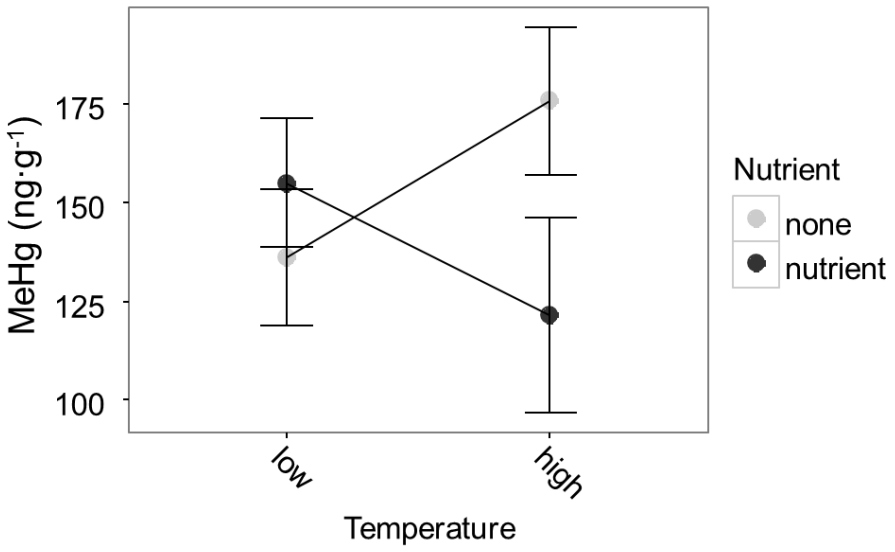


Figure 9. Interaction plot of averaged methylmercury concentrations in zooplankton ($ng \cdot g^{-1}$, dry weight) over both mid- and end-points of experiment, as influenced by temperature and nutrients. Error bars represent ±1 SE.

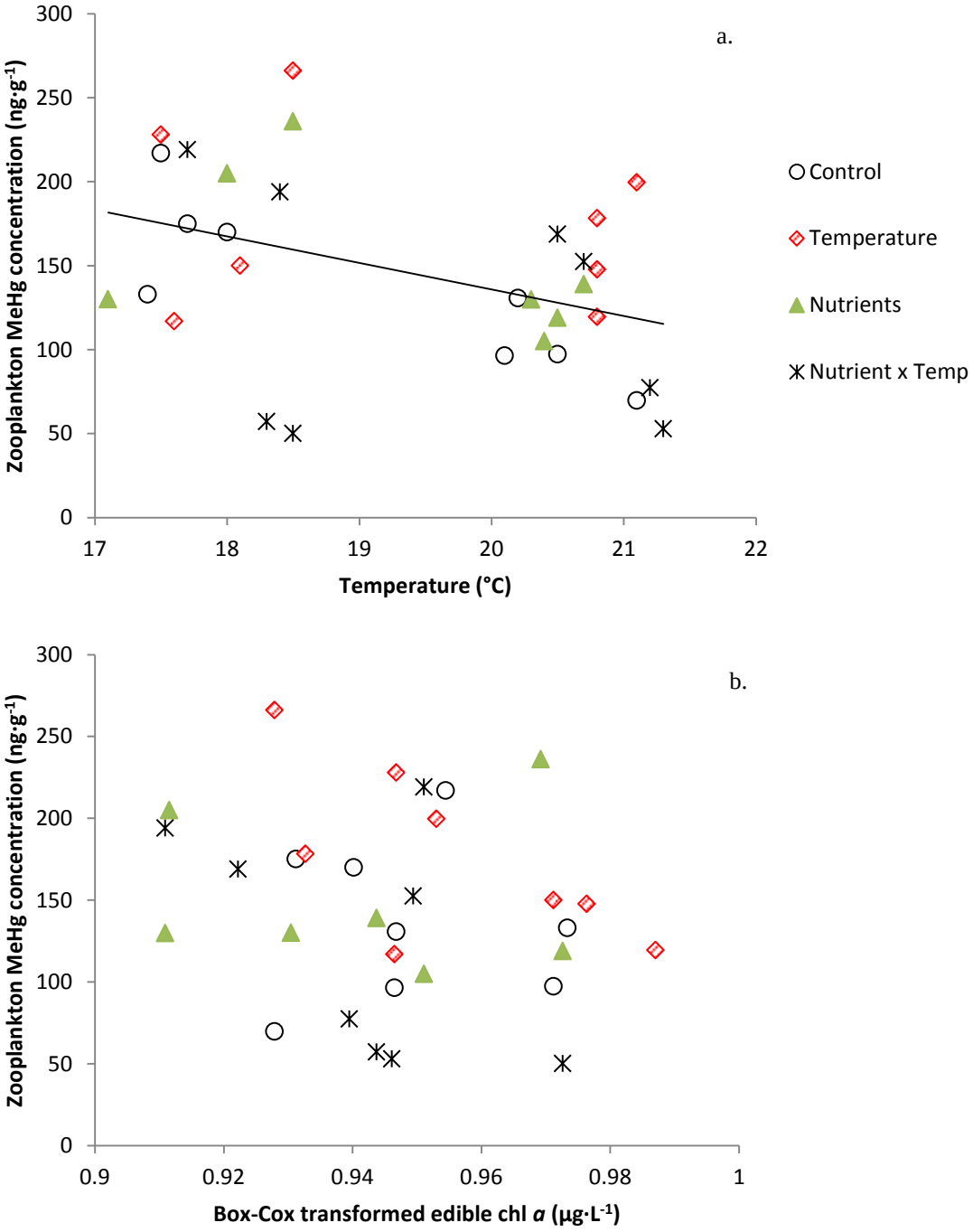


Figure 10. Regressions of average zooplankton MeHg concentrations (ng·g^{-1}) as a function of (a) temperature (°C) (y = -15.792x + 451.71) (R^2=0.16, p=0.024) and (b) edible chl *a* (µg·L^{-1}) (y = 404.24x - 237.18) (R^2=0.01, p=0.467) (N=32).

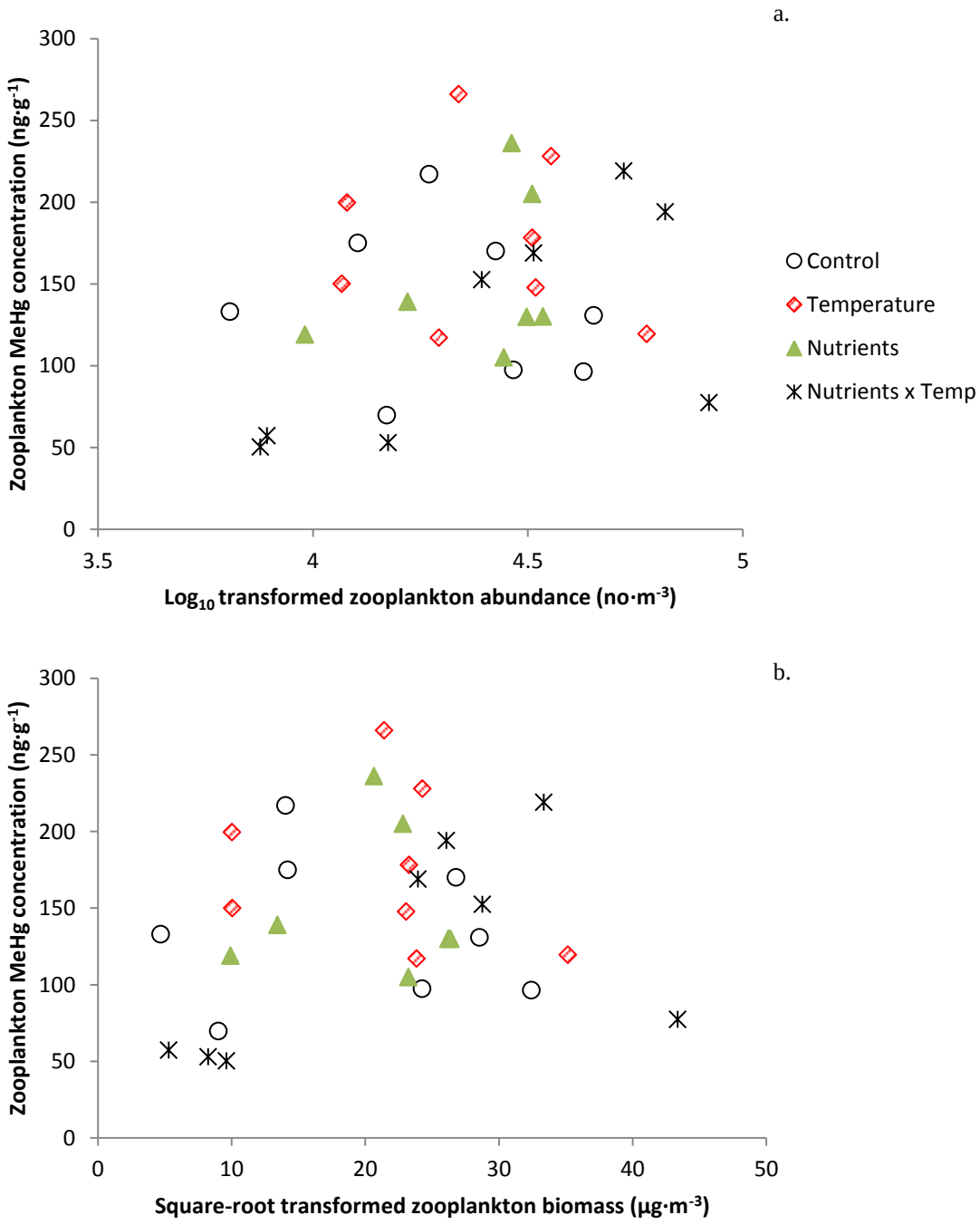


Figure 11. Regressions of average zooplankton MeHg concentrations (ng·g^{-1}) as a function of (a) zooplankton abundance (no·m^{-3}) (y = 48.872x - 67.67) (R^2=0.06, p=0.298) and (b) zooplankton biomass (µg·m^{-3}) (y = 0.9521x + 126.31) (R^2=0.03, p=0.391) (N=32).

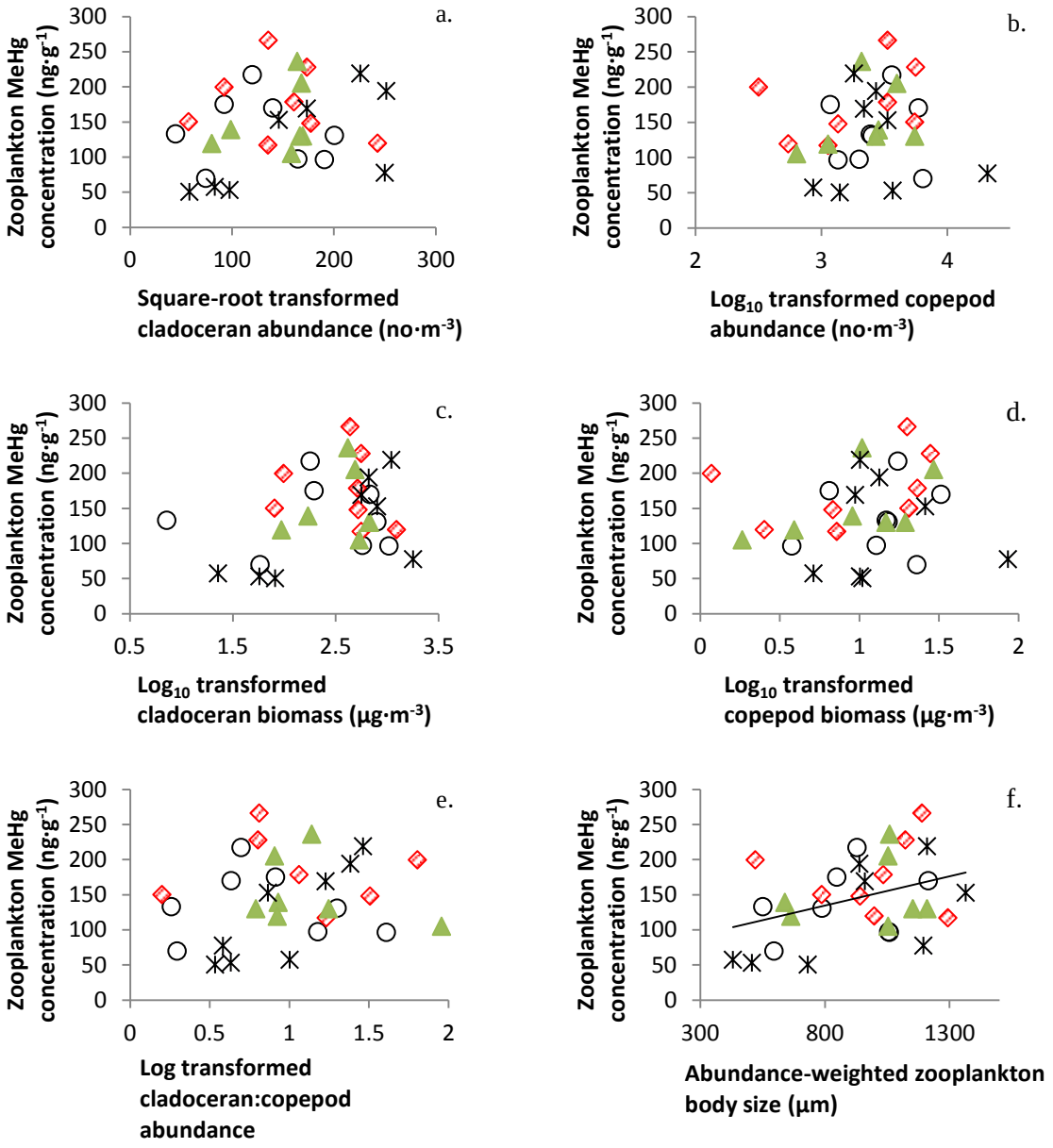


Figure 12. Zooplankton MeHg concentrations (ng·g^{-1}) as a function of (a,b) cladoceran and copepod abundance (no·m^{-3}) (y=0.2243x + 113.62, y=8.8703x + 116.3) (R^2=0.05, p=0.223, R^2=0.00, p=0.760) (c, d) cladoceran and copepod biomass(µg·m^{-3}) (y=28.956x + 74.259, y = 20.225x + 124.95)) (R^2=0.08, p=0.125, R^2=0.01, p=0.454) and (e,f) cladoceran:copepod abundance and abundance-weighted zooplankton body size (µm) (y = 13.962x + 131.78, y = 0.0831x + 68.152)) (R^2=0.01, p=0.475, R^2=0.14, p=0.038).

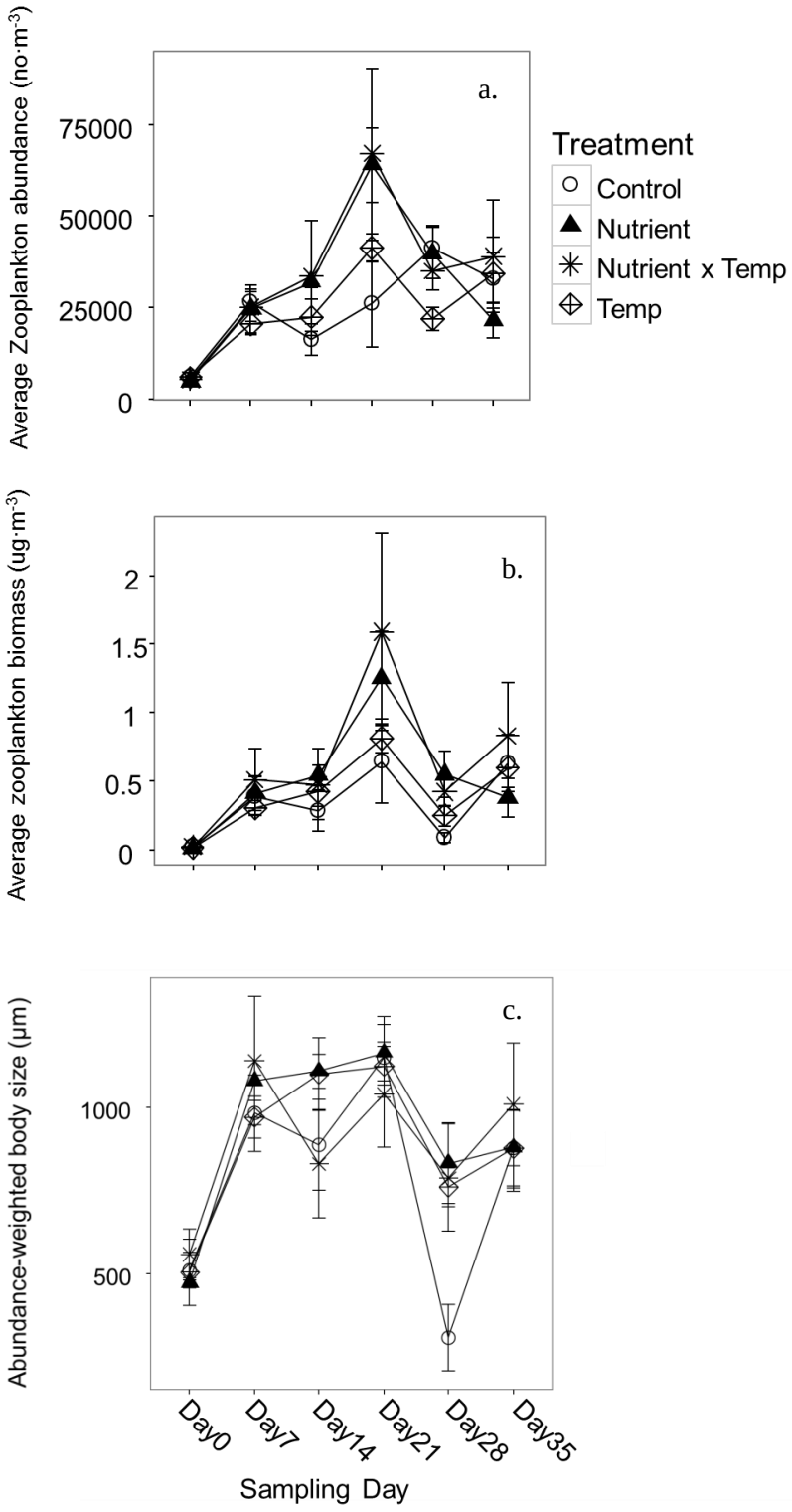


Figure 13. Total zooplankton metrics of (a) average abundance (no·m^{-3}), (b) average biomass (µg· m^{-3}) and (c) abundance-weighted body size (µm). Error bars represent ±1 SE.

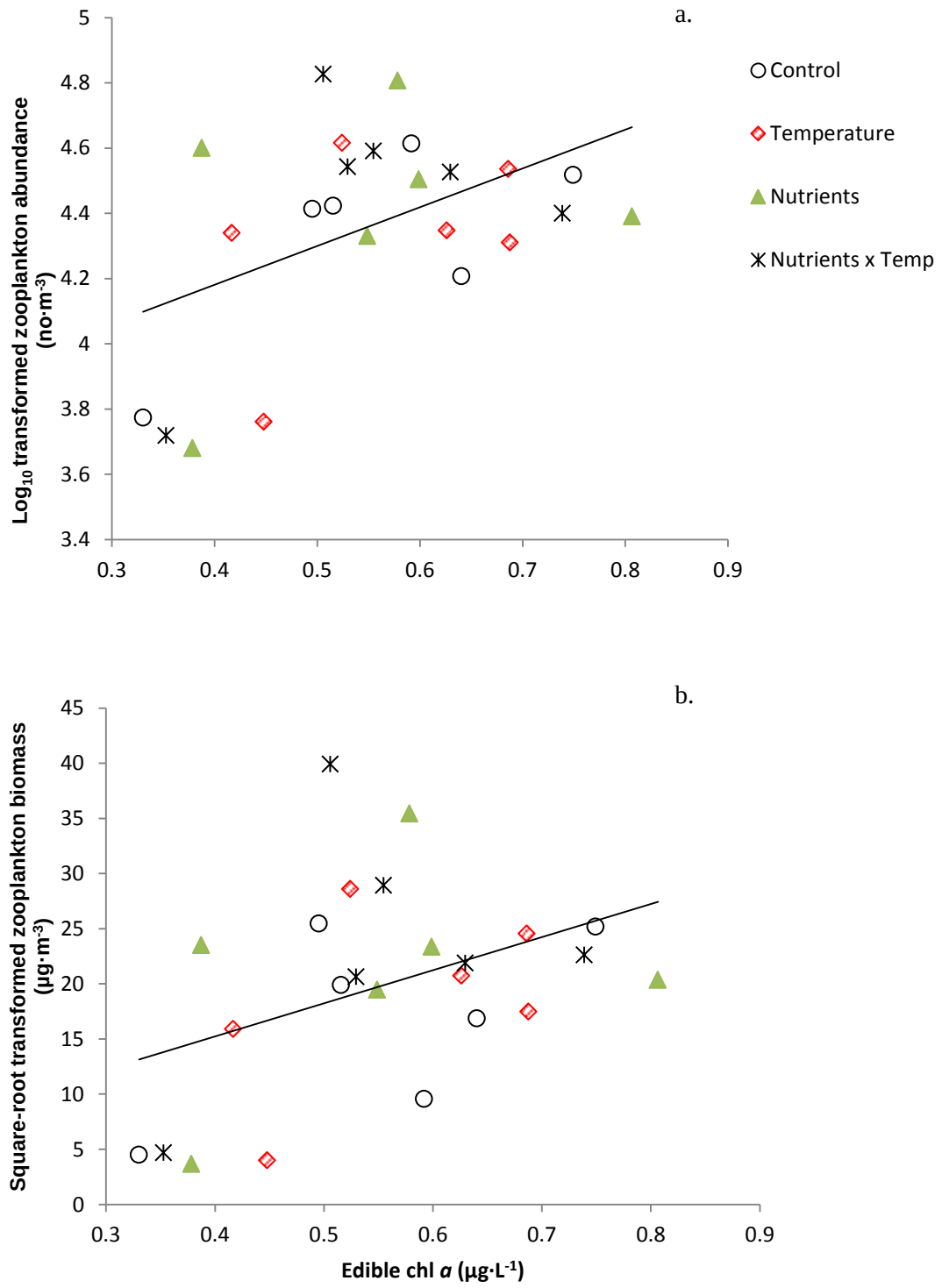


Figure 14. Regressions of averaged edible chl *a* (µg·L^{-1}) and (a) zooplankton abundance (no·m^{-3}) (y = 1.1893x + 3.7053)) (R^2=0.22, p=0.019) and (b) zooplankton biomass (µg· m^{-3}) (y = 30.034x + 3.2106) , R^2=0.17, p=0.046) (N=24).

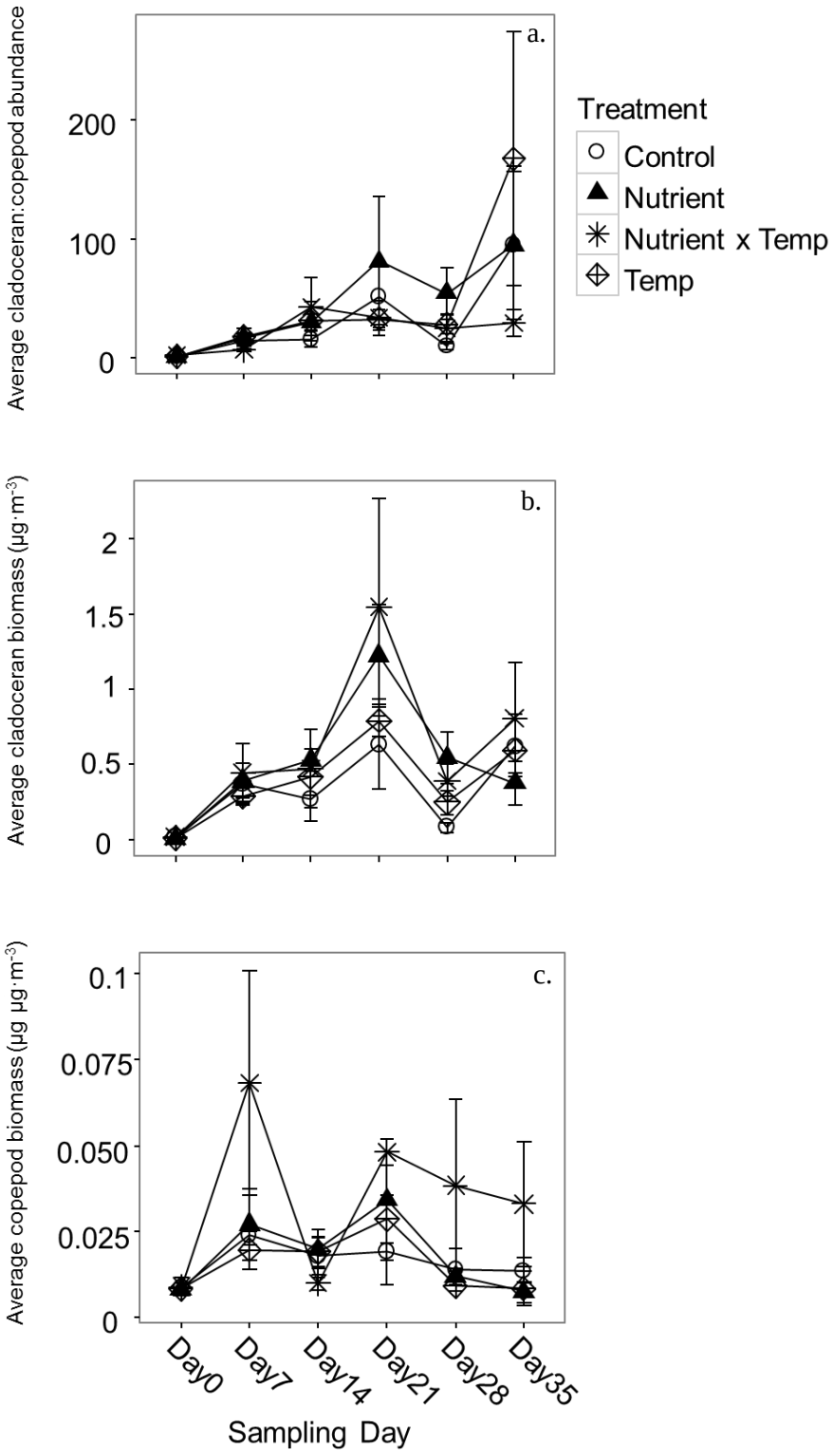


Figure 15. Average (a) cladoceran:copepod abundance, (b) cladoceran biomass ($\mu g \cdot m^{-3}$), and (c) copepod biomass ($\mu g \cdot m^{-3}$) by treatment and week of experiment. Error bars represent ±1 SE.

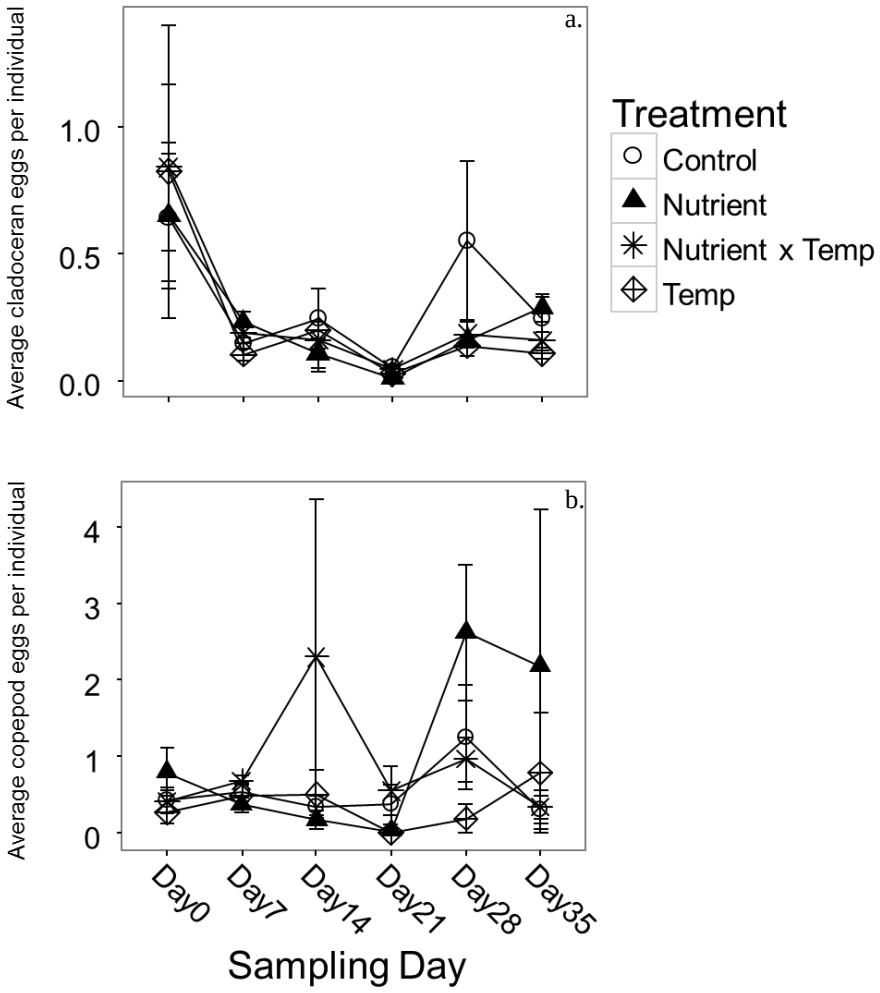


Figure 16. Average (a) cladoceran eggs and (b) copepod eggs per individual by treatment and week of experiment. Error bars represent ±1 SE.

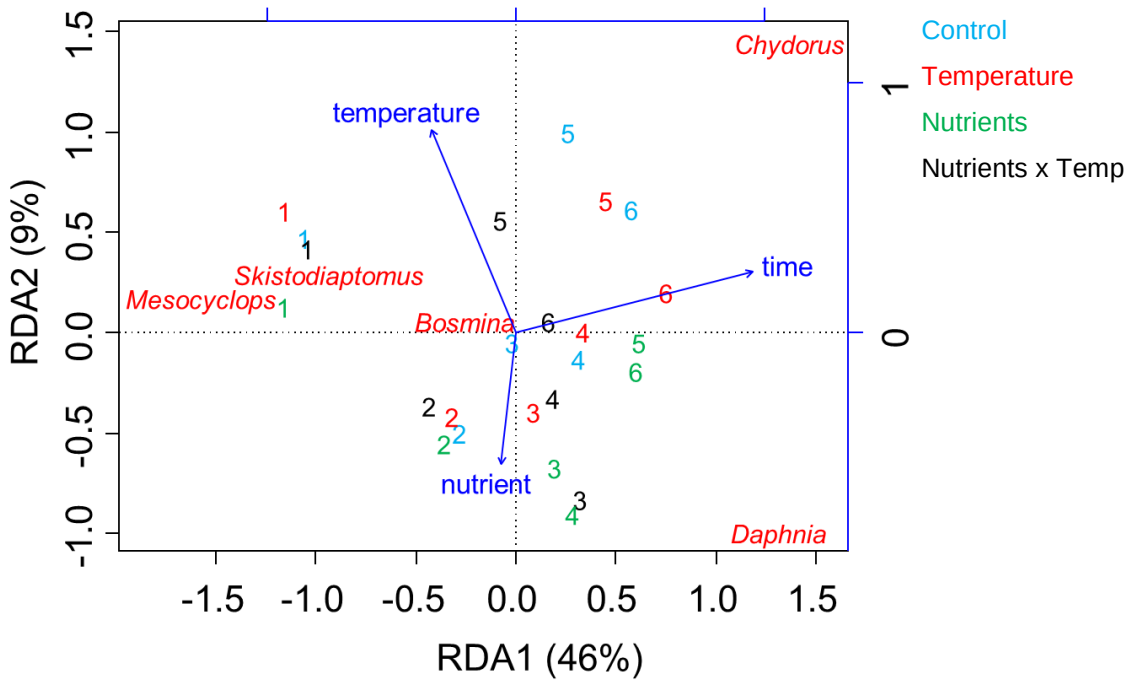


Figure 17. Redundancy analysis (RDA) plot showing influence of treatments and time on species abundances. Numbers represent the week of the experiment, and colors represent treatment combinations.

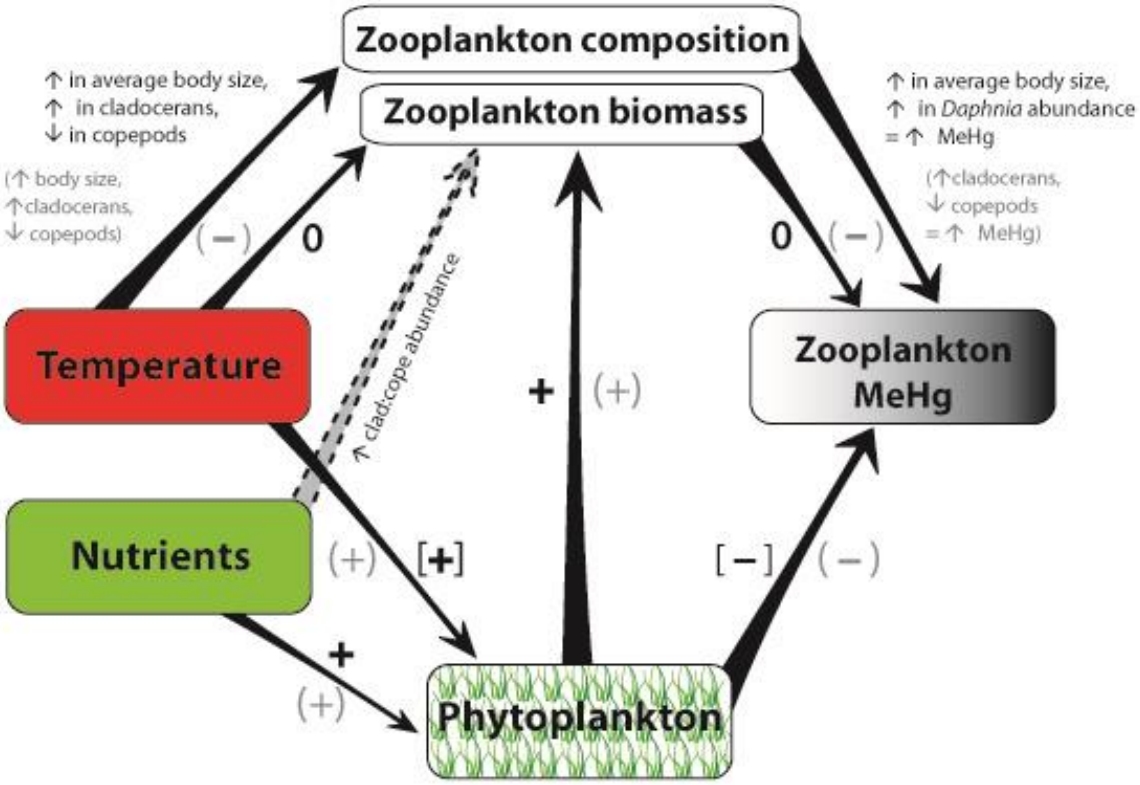
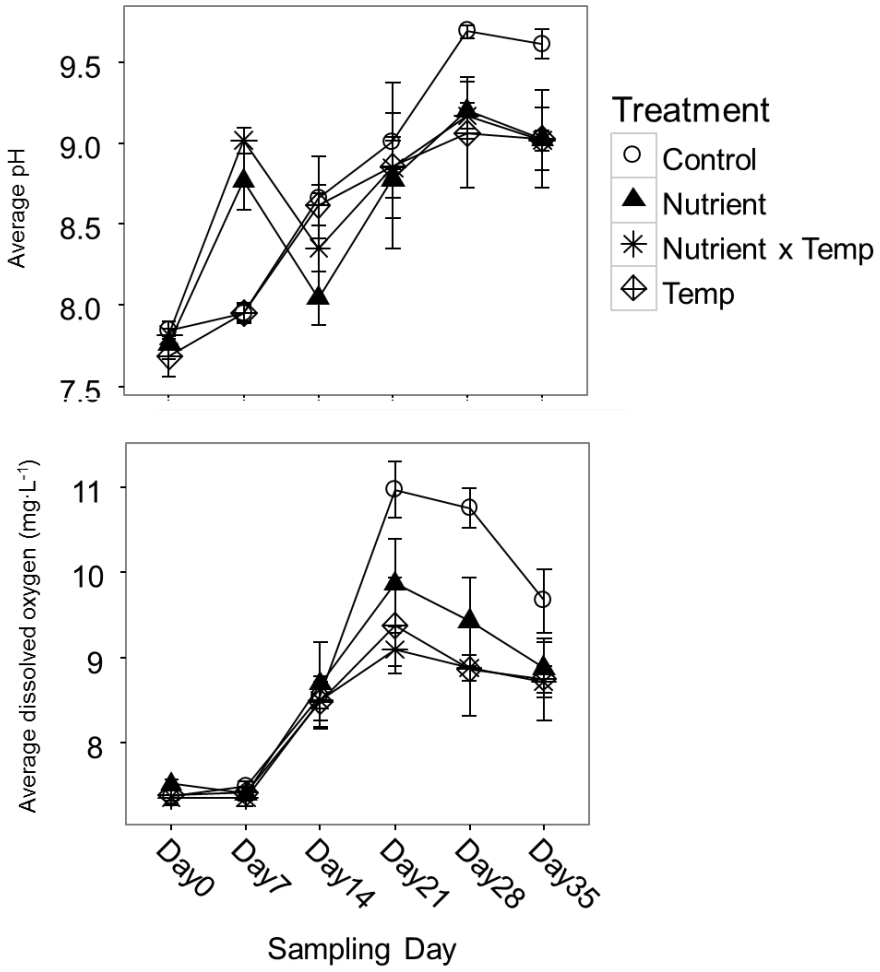


Figure 18. Results-based model, revised from conceptual model of predicted relationships between stressors (temperature and nutrients) and zooplankton, phytoplankton and zooplankton methylmercury in a freshwater system. Bolded symbols represent actual results, with weak relationships (p<0.10) in square brackets. Zeros indicate no relationship was found. Parenthetical grey symbols represent predicted results. Dashed arrow indicates an observed indirect relationship.

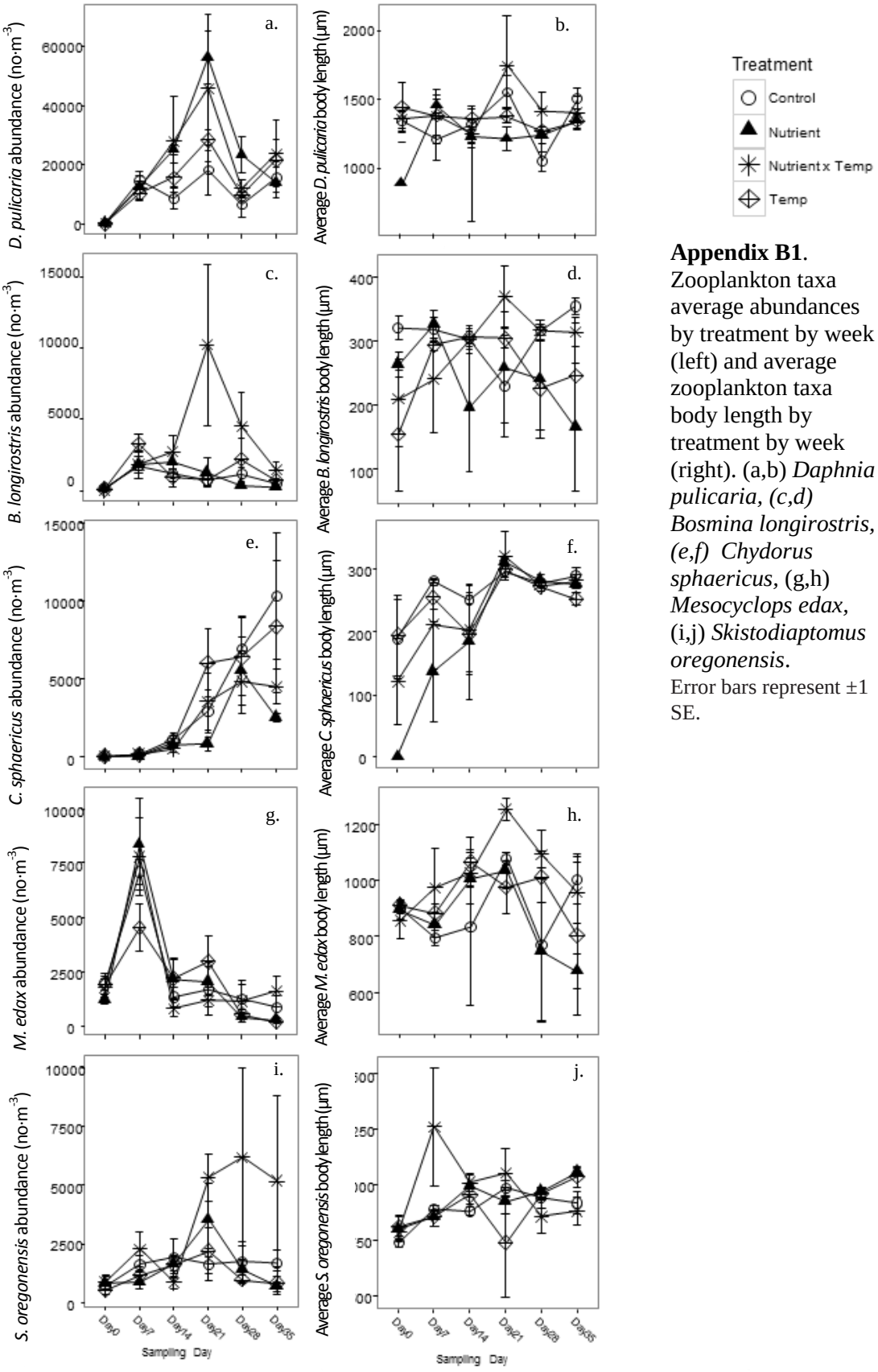
Appendices



Appendix A1. Average (a) pH and (b) dissolved oxygen ($mg \cdot L^{-1}$) by treatment combination by week. Error bars represent ±1 SE.

Appendix A2. Statistical summary of RM-ANOVA on water quality data of pH and dissolved oxygen ($mg \cdot L^{-1}$). Subscripts indicate degrees of freedom for RM-ANOVA. † $p<0.1$; * $p<0.05$.

Variable	Treatment	F ratio	*p* Value
pH	$Nutrient_{[1,12]}$	0.022	0.885
	$Temp_{[1,12]}$	0.274	0.611
	Nutrient x $Temp_{[1,12]}$	1.599	0.230
	$Time_{[4,48]}$	23.329	<0.001*
	Time x $Nutrient_{[4,48]}$	10.839	<0.001*
	Time x $Temp_{[4,48]}$	1.754	0.184
	Time x Nutrient x $Temp_{[4,48]}$	0.277	0.808
	$Nutrient_{[1,12]}$	1.636	0.225
DO	$Temp_{[1,12]}$	5.162	0.042*
	Nutrient x $Temp_{[1,12]}$	70.619	0.329
	$Time_{[4,48]}$	1.037	<0.001*
	Time x $Nutrient_{[4,48]}$	2.374	0.102
	Time x $Temp_{[4,48]}$	6.246	<0.001*
	Time x Nutrient x $Temp_{[4,48]}$	1.830	0.172



Appendix B1. Zooplankton taxa average abundances by treatment by week (left) and average zooplankton taxa body length by treatment by week (right). (a,b) *Daphnia pulicaria, (c,d) Bosmina longirostris, (e,f) Chydorus sphaericus,* (g,h) *Mesocyclops edax,* (i,j) *Skistodiaptomus oregonensis*. Error bars represent ±1 SE.

Appendix B2. Statistical summary of RM-ANOVA on zooplankton species data for weeks 2 – 5. Subscripts indicate degrees of freedom for RM-ANOVA. † $p<0.1$; * $p<0.05$.

Species	Treatment	Abundance *F* ratio	Abundance *p* value	Length *F* ratio	Length *p* value	Biomass *F* ratio	Biomass *p* value
D. pulicaria	$Nutrient_{[1,12]}$	3.353	0.092†	0.031	0.864	1.023	0.332
	$Temp_{[1,12]}$	0.661	0.432	0.025	0.877	0.007	0.935
	Nutrient x $Temp_{[1,12]}$	4.9	0.047*	2.182	0.165	0.026	0.875
	$Time_{[4,48]}$	0.555	0.642	1.089	0.362	0.571	0.629
	Time x $Nutrient_{[4,48]}$	2.103	0.12	0.399	0.729	1.216	0.318
	Time x $Temp_{[4,48]}$	0.107	0.952	0.689	0.547	0.451	0.708
	Time x Nutrient x $Temp_{[4,48]}$	0.761	0.52	0.571	0.616	0.766	0.514
S. oregonensis	$Nutrient_{[1,12]}$	0.690	0.423	0.246	0.629	2.875	0.116
	$Temp_{[1,12]}$	0.326	0.578	0.158	0.698	0.292	0.599
	Nutrient x $Temp_{[1,12]}$	0.843	0.246	0.014	0.909	0.004	0.953
	$Time_{[4,48]}$	0.611	0.630	1.240	0.309	1.246	0.308
	Time x $Nutrient_{[4,48]}$	1.486	0.490	0.699	0.574	1.418	0.255
	Time x $Temp_{[4,48]}$	0.135	0.952	0.593	0.643	1.788	0.170
	Time x Nutrient x $Temp_{[4,48]}$	0.658	0.600	0.287	0.856	2.525	0.077†
M. edax	$Nutrient_{[1,12]}$	0.882	0.366	1.722	0.214	0.728	0.410
	$Temp_{[1,12]}$	1.240	0.287	0.102	0.755	0.028	0.870
	Nutrient x $Temp_{[1,12]}$	0.004	0.948	0.697	0.420	1.392	0.261
	$Time_{[4,48]}$	0.862	0.470	3.372	0.024*	4.908	0.005*
	Time x $Nutrient_{[4,48]}$	1.827	0.159	3.304	0.026*	1.265	0.301
	Time x $Temp_{[4,48]}$	0.622	0.606	0.104	0.967	1.125	0.353
	Time x Nutrient x $Temp_{[4,48]}$	0.290	0.834	1.902	0.140	1.334	0.278
B. longirostris	$Nutrient_{[1,12]}$	1.000	0.337	0.134	0.721	0.331	0.576
	$Temp_{[1,12]}$	0.193	0.668	0.235	0.636	2.497	0.140
	Nutrient x $Temp_{[1,12]}$	1.859	0.198	1.341	0.269	2.074	0.175
	$Time_{[4,48]}$	0.248	0.855	0.237	0.855	3.243	0.031*
	Time x $Nutrient_{[4,48]}$	0.945	0.427	1.202	0.322	1.941	0.138
	Time x $Temp_{[4,48]}$	1.231	0.313	0.451	0.703	1.143	0.345
	Time x Nutrient x $Temp_{[4,48]}$	0.466	0.701	0.677	0.561	1.493	0.231
C. sphaericus	$Nutrient_{[1,12]}$	0.175	0.683	1.048	0.326	0.104	0.753
	$Temp_{[1,12]}$	0.016	0.903	2.030	0.180	0.005	0.946
	Nutrient x $Temp_{[1,12]}$	4.184	0.063†	0.934	0.353	2.235	0.161
	$Time_{[4,48]}$	1.824	0.157	6.548	0.001*	21.974	<0.001*
	Time x $Nutrient_{[4,48]}$	0.359	0.792	0.897	0.454	1.548	0.226
	Time x $Temp_{[4,48]}$	1.767	0.168	0.243	0.869	0.471	0.675
	Time x Nutrient x $Temp_{[4,48]}$	0.265	0.859	1.698	0.183	0.039	0.981