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Nutrients mediate the effects of temperature on methylmercury concentrations in freshwater zooplankton

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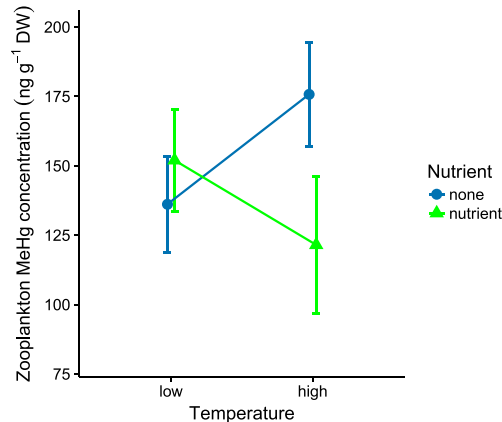
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HIGHLIGHTS

- High temperature treatments increased zooplankton methylmercury relative to controls.
- With warming, nutrients reduced zooplankton methylmercury compared to no nutrients.
- Responses were variable, likely due to the density of *Daphnia* and edible algae.
- A changing environment may alter methylmercury pathways in freshwater ecosystems.

GRAPHICAL ABSTRACT



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ABSTRACT

Methylmercury (MeHg) bioaccumulation in freshwater aquatic systems is impacted by anthropogenic stressors, including climate change and nutrient enrichment. The goal of this study was to determine how warmer water temperatures and excess nutrients would alter zooplankton communities and phytoplankton concentrations, and whether those changes would in turn increase or decrease MeHg concentrations in freshwater zooplankton. To test this, we employed a 2 × 2 factorial experimental design with nutrient and temperature treatments. Mesocosms were filled with ambient 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 0.7 °C), nutrient addition (a single pulse of 10× ambient concentrations of nitrogen and phosphorous), control, and a combination of temperature and nutrients were applied to mesocosms. The individual treatments altered phytoplankton densities and community structure, but alone the effects on MeHg concentrations were muted. Importantly, we found a significant interactive effect of nutrients and temperature: the nutrient addition appeared to buffer against increased MeHg concentrations associated with elevated temperature. However, there was variability in this response, which seems to be related to the abundance of *Daphnia* and edible phytoplankton. Nutrients at low temperature were associated with marginal increases (1.1×) in zooplankton MeHg. Our findings suggest that global change drivers that influence

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community composition and ecosystem energetics of both zooplankton and phytoplankton can alter MeHg pathways through food webs.

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1. Introduction

Freshwater ecosystems currently face a growing diversity of human-induced stressors. Inputs of persistent pollutants like mercury (Hg) are particularly concerning because they are widespread, can negatively affect organisms in the environment, and pose human health risks through food web bioaccumulation (Meybeck and Vörösmarty, 2005; Mergler et al., 2007; Eagles-Smith et al., 2018). Other anthropogenic stressors, such as climate change and eutrophication, can further 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; Walters et al., 2015; Eagles-Smith et al., 2018). As Hg is primarily accumulated through dietary pathways, it is critical to understand how freshwater organisms mediate the transfer of Hg to higher trophic levels (Fitzgerald and Mason, 1997; Kuwabara et al., 2005; Eagles-Smith et al., 2008; Stewart et al., 2008). With the growing recognition that other anthropogenic stressors interact with Hg to influence bioaccumulation, it is critical to better quantify the mechanisms driving bioaccumulation.

Freshwater systems are particularly vulnerable to the cascading effects of climate change. Altered temperature regimes in freshwater systems can shift the composition of phytoplankton communities (Butler et al., 1989; Paerl et al., 2011), influence zooplankton diversity, body size, and fecundity (Moore and Folt, 1993; Chen and Folt, 1996; Weetman and Atkinson, 2004), and impact higher level consumers through changes in food quality and availability (Chen and Folt, 1996). Each of these processes can influence Hg bioaccumulation through freshwater food webs in complex ways because Hg in higher trophic levels is obtained largely through dietary pathways. For example, warmer water temperatures can 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 (Dijkstra et al., 2013). Conversely, warmer temperatures may also increase growth rates, diluting Hg in the body, and resulting in a lower overall Hg concentration (Ward et al., 2010).

The exponential rise of modern agriculture since the Industrial Revolution has also impacted aquatic systems. Nutrient-rich runoff from agricultural areas changes aquatic food-web dynamics through eutrophication and shifts in primary productivity (Smith et al., 1998; Ramankutty and Foley, 1999). Excess nutrients commonly stimulate production of phytoplankton species that are largely inedible to freshwater zooplankton, thus nutrient enrichment impacts both food availability and quality (Vitousek et al., 1997; Correll, 1998; Brett et al., 2000; Paerl et al., 2011), resulting 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). Zooplankton may also be able to consume some of the increased primary productivity and therefore may respond positively, but in general, they may not be able to keep phytoplankton biomass in check because of an abundance of inedible species (e.g., Vanni and Temte, 1990).

Nutrient-induced primary productivity has ramifications for how mercury moves through aquatic food webs. There is still ambiguity about whether highly productive systems will increase mercury bioaccumulation by promoting mercury methylation via a supply of increased labile organic material, or whether such eutrophic systems will buffer higher trophic levels from mercury uptake through biodilution. Biodilution occurs when a proliferation of algae dilutes available MeHg at the base of the food web, before it can get to higher consumers (Pickhardt et al., 2002; Chen and Folt, 2005; Walters et al.,

2015). The methylation of mercury is generally system-dependent, requiring specific biogeochemical conditions that often occur in managed ecosystems such as wetlands and reservoirs, where temperature and primary productivity are some of the key factors in this process (Appendix A) (Eagles-Smith et al., 2008; Stewart et al., 2008; Dijkstra et al., 2013). In mercury-contaminated waterbodies, understanding the interplay between factors that control mercury methylation and those that influence methylmercury bioaccumulation can inform management decisions to reduce the health risks to fish, wildlife, and humans posed by methylmercury.

Nutrient loading and warmer temperatures in aquatic systems can both influence MeHg concentrations, as well as induce shifts in zooplankton community composition, which may indirectly change MeHg concentrations in higher level predators (Winder et al., 2009). High zooplankton abundance has been shown to negatively correlate with MeHg concentrations in fish (Chen and Folt, 2005), but other studies have found that seasonal differences in zooplankton species composition may alter the relationship between zooplankton abundance and fish MeHg concentrations (Watras and Bloom, 1992; Kuwabara et al., 2005). It is likely that the mechanisms of MeHg bioaccumulation are a function of the species and system properties (i.e., context dependent). Kainz et al. (2006) found that larger zooplankton body size, not identity, was critical in estimating potential MeHg concentrations of herbivorous zooplankton communities. In contrast, others have found that particular zooplankton orders, such as copepods or cladocerans, can influence bulk MeHg concentrations of the zooplankton community 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). 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). Thus, zooplankton community structure, and the factors that influence it, are important considerations in quantifying movement of MeHg through an aquatic food web.

The purpose of this study was to examine the possible effects of two common indicators of environmental change, elevated temperature and nutrients, on both the community composition and the mercury concentrations of zooplankton using organisms from a mercury-contaminated reservoir. Through manipulative mesocosm experiments we tested whether: (1) the combination of these stressors reduced MeHg concentrations in zooplankton through shifts in zooplankton community structure, (2) elevated water temperatures changed accumulation of MeHg in zooplankton, and (3) nutrient additions interacted with MeHg accumulation in zooplankton through biodilution. Over the course of five weeks, our experiment demonstrates that warming and nutrients can interact to affect zooplankton MeHg accumulation, with zooplankton community composition playing an important role.

2. Methods

2.1. Study site

The experiment took place at Cottage Grove Reservoir, Oregon (latitude: 43°43'00", longitude: 123°02'55"), which is located at river mile 29 of the Coast Fork of the Willamette River, approximately 15 km downstream of the Black Butte Mine (Appendix A). The mine was a site of historical Hg mining and abandoned mine tailings have led to its designation as a US EPA Superfund site. The reservoir is 469 ha at

full pool and 22 m deep, with an average depth of 9 m (Johnson et al., 1985). The reservoir is dilute (conductivity = 63 $\mu\text{mhos cm}^{-1}$), circumneutral (pH 7.7), and is considered mesotrophic (Johnson et al., 1985). The reservoir has been subject to a fish consumption advisory since 1979, nearly ten years after the mine closed (Curtis et al., 2013). Fish tissue THg concentrations have been consistently $>0.3 \text{ mg kg}^{-1}$ ww in Cottage Grove Reservoir, with some values as high as 2.5 mg kg^{-1} (Hope and Rubin, 2005; Curtis et al., 2013). Samples taken from the reservoir in summer 2013 found whole water MeHg concentrations averaged 0.10 ng L^{-1} (range: $0.07\text{--}0.12 \text{ ng L}^{-1}$) and filtered ($0.45 \mu\text{m}$) water MeHg averaged 0.08 ng L^{-1} (range: $0.06\text{--}0.12 \text{ ng L}^{-1}$) (Eckley et al., 2015).

2.2. Experimental set-up

To test our objectives, we used a 2×2 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 polyethylene cattle watering tanks ($132.08 \text{ cm} \times 78.11 \text{ cm} \times 60.96 \text{ cm}$; 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 1 m at the boat ramp on 12 July 2013. Water was then pumped into the mesocosms after filtering through $10\text{-}\mu\text{m}$ nylon mesh to remove large sediments and screen out large phytoplankton and zooplankton. The water in these mesocosms was not amended with reservoir water after the initial filling and reservoir sediments were not added to the mesocosms following Pickhardt et al. (2002), as sediment with unknown and unstandardized metal concentrations could have confounded our results. Thus, there was not a renewable source of MeHg, as would be observed in a natural system. However, we contend that in an experiment of relatively short duration, the main effect of excluding renewable MeHg is to make our results on zooplankton MeHg concentrations more conservative. Further, our goal was not to perfectly recreate a natural system, but rather to compare relative changes between treatments to elucidate potential mechanisms of mercury bioaccumulation.

On 16 July 2013, mesocosms were inoculated with reservoir phytoplankton and zooplankton, collected by vertical tows from the reservoir's epilimnion (0–4 m) using a 30-cm diameter plankton net with $80\text{-}\mu\text{m}$ mesh. Plankton were transported using 5-L carboys and deposited into mesocosms promptly after collection. Reservoir zooplankton density was estimated by sampling the reservoir at five locations at depths of 3 m, 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 mesocosms. This allowed us to collect individuals that were not undergoing diel vertical migration, which could alter accumulation of MeHg. 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, mesocosms were inoculated at $1.5\times$ the ambient reservoir zooplankton density. Mosquito larvae and mites, which prey on zooplankton and could alter zooplankton densities, were removed by hand. All mesocosms were then covered with mosquito netting to minimize mosquito breeding and other invertebrate colonization within the mesocosms, and left to equilibrate for 48 h before treatments began.

On 18 July 2013, the temperature treatment was applied using custom-built passive greenhouse canopies following Strecker et al. (2004). Greenhouse canopies were used to passively warm temperature treatment mesocosms approximately $0.5 \text{ }^\circ\text{C}$ compared to control mesocosms, a conservative and near-future representation of climate change in the Pacific Northwest (Mote and Salathé, 2010). Canopies were constructed using PVC pipe, Tufflite IV greenhouse sheeting (6

mil [0.15 mm] thickness), and plastic louvered dryer vents. Greenhouse sheeting of this type has high transmission of photosynthetically active radiation (90%) but reduced UV transmission because of additives used to protect the sheeting from degradation from UV radiation (Papadakis et al., 2000). All mesocosms were covered by these canopies to control for solar radiation. Temperature treatments had canopies lowered to sit on edge of mesocosms, and all vents were closed. Control mesocosms had canopies raised approximately 25.4 cm off of mesocosm edge, and all vents were opened. At each weekly temperature sampling, vents were closed or opened to adjust for desired temperature based on treatment.

Nutrient treatments were also applied on 18 July 2013. Nutrient mesocosms 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 L^{-1} and 0.013 mg L^{-1} , respectively (US Army Corps of Engineers, 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 mesocosms were stirred manually to distribute nutrients; all mesocosms were stirred to control for any unintended effects caused by the water disturbance.

2.3. Sampling and sample processing

Sampling of the reservoir and the experimental mesocosms occurred weekly for five weeks, beginning 18 July (Day 0) to 22 August 2013 (Day 35). Day 0 sampling took place before treatments were applied. Mesocosm zooplankton samples were collected by taking a 22-L water sample with a Van Dorn sampler, followed by filtration with $80\text{-}\mu\text{m}$ mesh. Water was returned to mesocosms after zooplankton sample removal. Zooplankton samples were stored at a final solution of 70% ethanol. Zooplankton abundance was estimated by 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), which allows more adults to be counted and identified to species in situations where juveniles are numerous. 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 mesocosm for all five weeks were measured and averaged. Length-weight regressions were used to estimate biomass by using the average length of 10 individuals per taxa per sample (McCauley, 1984; Culver et al., 1985; Lawrence et al., 1987).

Zooplankton samples were taken from both the experimental mesocosms and the reservoir, and analyzed for total and methylmercury at the experiment start, middle and end (Days 0, 14, and 35). These dates were chosen for two reasons: 1) more frequent sampling would have greatly reduced zooplankton biomass; 2) zooplankton development times can range from one to several weeks at these temperatures, thus these dates encompass at least one generation. These samples were collected following the EPA Method 1631 "clean hands/dirty hands" techniques for mercury tissue sample collection (US EPA, 2002). Zooplankton samples were collected from the reservoir by vertical plankton tows using a 30-cm diameter plankton net with $80\text{-}\mu\text{m}$ mesh from 3 m above the lake bottom to water surface. Zooplankton samples were 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. We used trace-element certified acid-washed amber borosilicate glass bottles that were acid cleaned with nitric acid since we were sampling zooplankton and not water, which is more sensitive to contamination.

Mesocosm zooplankton samples required multiple grabs with a Van Dorn sampler and subsequent filtration due to the size limitations of the mesocosms; sample collection methods were otherwise identical to reservoir methods. Though removing zooplankton from mesocosms could affect subsequent results, we took the minimum amount needed to have enough biomass for mercury analyses (~1 mg dry weight, equivalent to 5–12% of total zooplankton biomass, depending on the date). We observed an increase in zooplankton abundance and biomass the week following removal (i.e., Day 21), suggesting that the effect of removal was minimal. The size of the mesocosms precluded sorting zooplankton into more refined taxonomic groups, as there was not enough biomass for these analyses. Zooplankton samples were freeze-dried and homogenized with a ceramic mortar and pestle, acid washed before each sample with 5% ultrapure HNO₃ followed by a DI water rinse. 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. Recoveries for continuing calibration verification standards (CCVs) were 105.1% ($n = 7$) for MeHg and 105.9% for THg ($n = 3$), using MeHgCl and HgCl, respectively. Certified reference material (CRMs, TORT-3) recoveries were 104.2% ($n = 11$) for MeHg and 109.4% ($n = 2$) for THg, with a standard deviation = 9.42%. Relative percent difference averaged 3.64% for all MeHg duplicates, and 2.54% for THg. Matrix spike recoveries ($n = 2$) for MeHg averaged 117%.

Water for chlorophyll *a* (chl *a*) analysis was taken weekly using grab samples from the mesocosms and the reservoir, using 1-L amber bottles. These water samples were stored on ice in a cooler, then processed on site within 2–3 h 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 highly edible fraction of chl *a* only (Cyr and Curtis, 1999); 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), which were then frozen until analysis in the lab. Filters were soaked in acetone and refrigerated for 20 h 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 mesocosms. Temperature and dissolved oxygen were also measured at 1-m intervals in the reservoir, and pH was measured at the water surface. Results for pH and dissolved oxygen are reported in Appendix B.

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 phosphorous were taken using grab samples from the mesocosms and the reservoir, which were put on dry ice shortly after collection and then completely frozen until analysis. On Day 0, only the reservoir and the nutrient treatment mesocosms were sampled (post-nutrient addition) as the nutrient concentrations in the reservoir were representative of the non-nutrient addition mesocosms at the experiment start. On Day 35, all 16 mesocosms and the reservoir were sampled for nutrient concentrations. Total nitrogen samples were analyzed at Oregon State University's Cooperative Chemical Analytical Laboratory following CCAL 33A.3 method (Cooperative Chemical Analytical Laboratory (CCAL), 2013). Total phosphorous samples were processed

using the CCAL 35B.2 method (Cooperative Chemical Analytical Laboratory (CCAL), 2010), and then analyzed using a Shimadzu UV-1800 Spectrophotometer (Shimadzu, Kyoto, Japan).

2.4. Statistical analyses

The primary objective of this study was to examine the singular and interactive effects of nutrients and temperature on zooplankton mercury concentrations, total and edible chlorophyll *a*, and zooplankton community metrics. Two-factor repeated measures analysis of variance (RM-ANOVA) and two-factor ANOVA were run with fixed effects of treatments (nutrients, temperature). Treatments were applied on Day 0 and therefore the first week was not included in analyses. Environmental variables (temperature, dissolved oxygen, pH) 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. Environmental criteria were also compared between treatments using two-factor RM-ANOVA to examine any possible confounding factors. 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. Variables were transformed when assumptions were violated. Greenhouse-Geisser corrections (when $\epsilon < 0.75$) were used when the assumption of sphericity was violated. Analyses were performed using the libraries EZ (Lawrence, 2013) and stats in R version 3.1.2 (R Core Team, 2014).

3. Results

3.1. Environmental conditions

Over five weeks we achieved our goal of ~0.5 °C differences between treatments. Temperature mesocosms were significantly warmer 20.3 °C ($\pm 0.55\text{SD}$) than mesocosms without the temperature treatment 19.6 °C ($\pm 0.53\text{SD}$) (Appendix B). Reservoir surface temperatures were consistently warmer than all mesocosms, though general warming and cooling trends tracked similarly between the mesocosms and the reservoir (Appendix B).

The second factor of this experiment was a nutrient treatment. The nutrient addition on Day 0 effectively raised nutrient levels in nutrient treatment mesocosms (Appendix B). Total nitrogen (TN) was, on average, 1.8 \times higher in treatment mesocosms on Day 7, averaging 0.55 mg L⁻¹, compared to water from Cottage Grove Reservoir (0.3 mg L⁻¹) ($F_{2,6} = 17.47, p = 0.003$). Total phosphorous (TP) concentrations were also significantly greater in the nutrient treatment (0.080 mg L⁻¹) compared to the reservoir concentrations (0.006 mg L⁻¹) following the nutrient addition on Day 7, averaging 14 \times higher concentrations than the reservoir ($F_{2,6} = 19.36, p < 0.001$). These levels of total nitrogen and phosphorous in the treatment mesocosms are considered eutrophic, thus achieving the desired treatment (Wetzel, 2001).

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 mesocosms, and equilibrated by Day 14 (Fig. 1). The edible fraction (<35 μm) was significantly impacted by time and the interaction of time and nutrients, showing an average 1.2 \times increase over edible chl *a* in nutrient treatment mesocosms as compared to mesocosms without nutrient additions (Table 1). However, on Day 7, this effect was much greater, with 3 \times more edible chl *a* in nutrient mesocosms compared to no nutrient mesocosms. Treatments did not have a significant effect on total chl *a*, though there was a weak positive effect of temperature over time (Table 1). Both edible and total chl *a* in the experiment were consistent with average reservoir concentrations

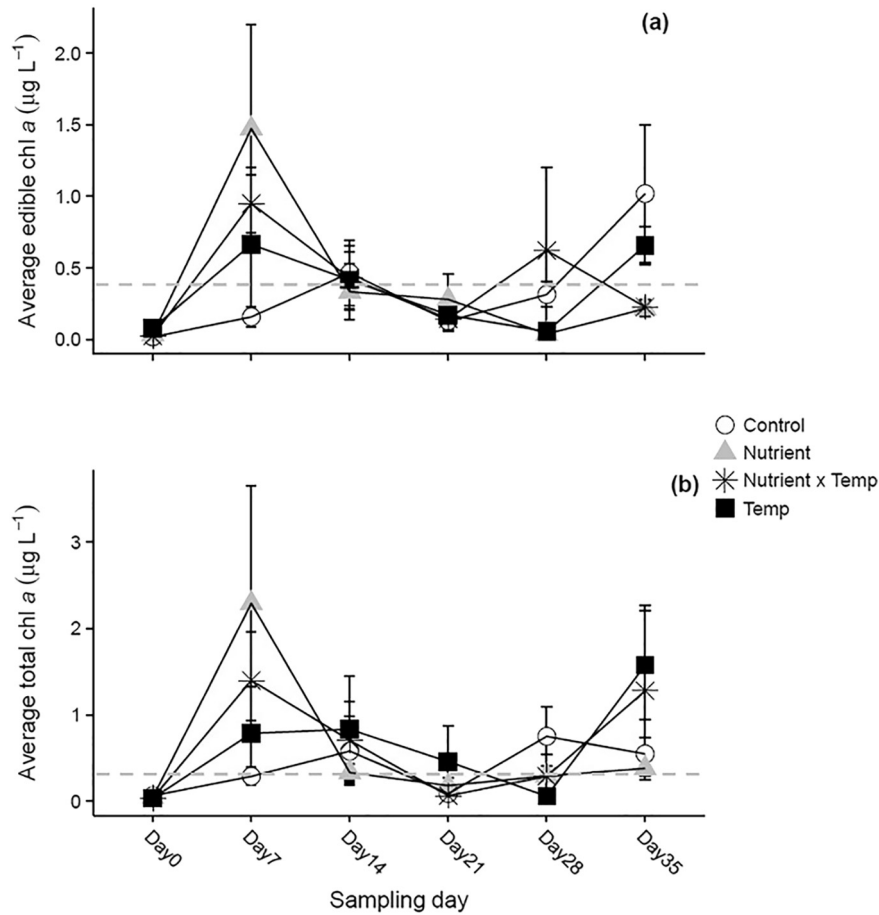


Fig. 1. Average (a) edible and (b) total chlorophyll *a* ($\mu\text{g L}^{-1}$) by treatment combination by week. Error bars represent ± 1 SE. Dashed grey line represents average chl *a* concentrations in Cottage Grove Reservoir through the experiment.

(dashed line in Fig. 1). Though the nutrient concentrations were indicative of eutrophic conditions, chlorophyll *a* concentrations were more representative of oligotrophic conditions, suggesting that our nutrient treatments had modest effects on phytoplankton biomass.

3.2. Zooplankton community and species metrics

Zooplankton community metrics were highly variable and showed mixed results in response to treatments (Fig. 2). Although not significant at $\alpha = 0.05$, nutrients and temperature increased zooplankton abundance by 1.3 \times and 1.1 \times , respectively, compared to the control over the last five weeks of the experiment at $\alpha = 0.10$ (Fig. 2, Table 2). Biomass and average body length showed no significant effects of treatments (Fig. 2, Table 2). However, when temperature is treated as a continuous variable, we observed a modest negative relationship

between temperature and biomass ($R^2 = 0.058$, $p = 0.019$). There was a weakly positive relationship of % edible chlorophyll *a* and both cladoceran ($r = 0.202$, $p = 0.051$) and total zooplankton biomass ($r = 0.200$, $p = 0.054$) at $\alpha = 0.10$. There were significant positive correlations ($\alpha = 0.05$) of both edible and total chl *a* with copepod biomass (edible: $r = 0.206$, $p = 0.046$; total: $r = 0.253$, $p = 0.014$).

Community composition was assessed to see if shifts in the relative abundance of different taxa could potentially explain differences in MeHg concentrations. At the start of the experiment, there were roughly even proportions of cladocerans and copepods. The ratio of cladoceran:copepod biomass was significantly impacted by the interaction of nutrients and temperature over time: by experiment end, temperature treatments with no nutrients were entirely dominated by cladocerans (160 \times more cladocerans than copepods), whereas temperature treatments with nutrients were still dominated by cladocerans, but to a lesser extent (29 \times more cladocerans than copepods) (Fig. 2, Table 3). However, biomass of cladocerans and copepods were unaffected by treatments (Fig. 2, Table 3). Again, when temperature is treated as a continuous variable, we observed a modest negative relationship between temperature and cladoceran biomass ($R^2 = 0.061$, $p = 0.017$). The five dominant species of zooplankton found in the mesocosms and in the reservoir were the cladocerans *Daphnia pulicaria*, *Bosmina longirostris*, and *Chydorus sphaericus* and the copepods *Mesocyclops edax* and *Skistodiaptomus oregonensis*. The interaction of nutrients \times temperature affected *D. pulicaria*: abundance increased in the presence of both stressors relative to the control (Appendix C), with nutrients generally increasing abundance relative to no nutrient treatments. There were no significant treatment effects for the other species (Appendix C).

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

Treatment	Chl <i>a</i> , edible		Chl <i>a</i> , total	
	F-ratio	p-Value	F-ratio	p-Value
Nutrient _[1,12]	0.001	0.980	0.052	0.824
Temp _[1,12]	0.132	0.723	0.189	0.671
Nutrient \times Temp _[1,12]	0.055	0.819	0.005	0.942
Time _[4,48]	5.387	0.001*	12.196	<0.001*
Time \times Nutrient _[4,48]	3.392	0.016*	1.575	0.196
Time \times Temp _[4,48]	1.043	0.395	2.241	0.078†
Time \times Nutrient \times Temp _[4,48]	0.741	0.569	1.263	0.297

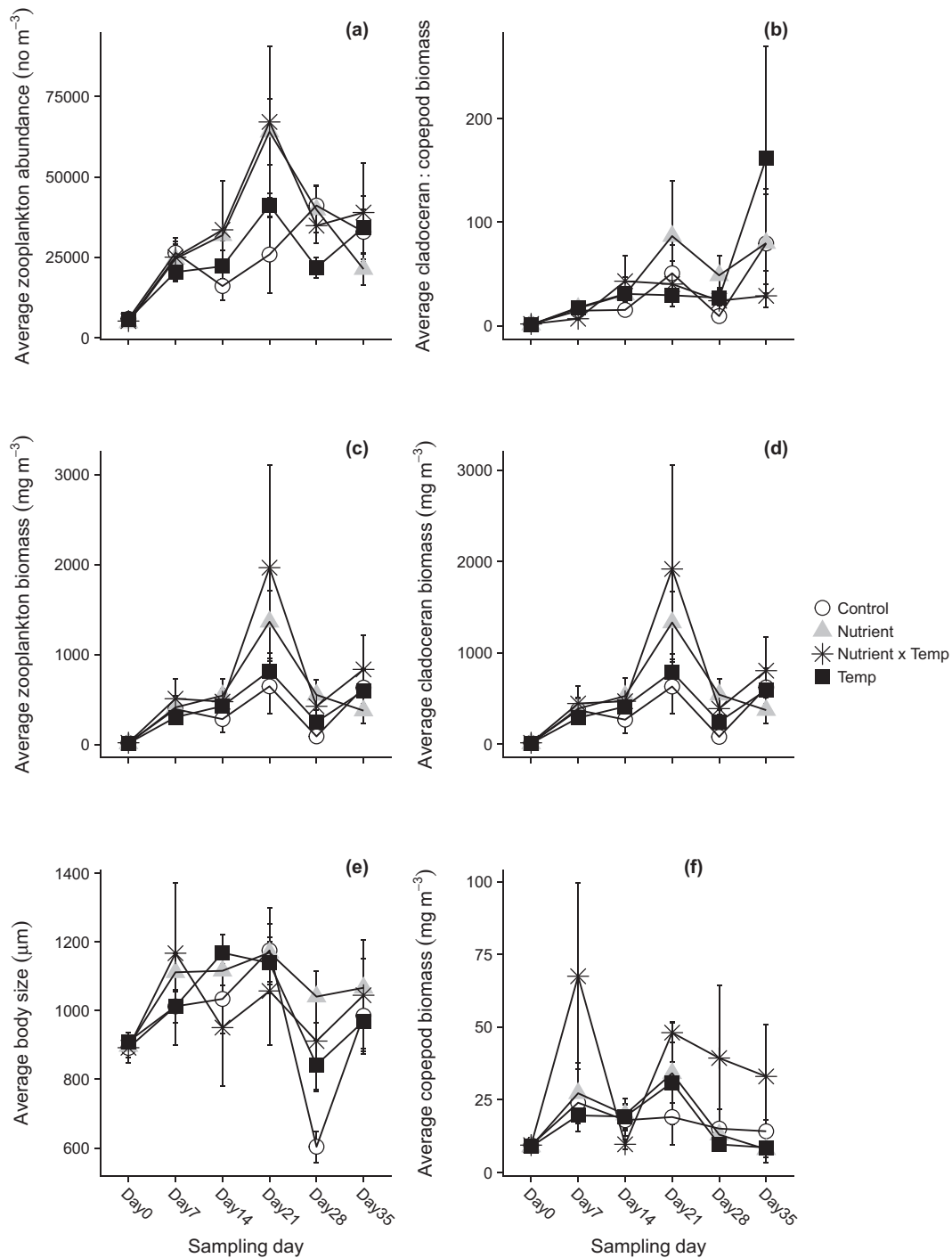


Fig. 2. Average (a) zooplankton abundance (no m⁻³), (b) cladoceran:copepod biomass, (c) zooplankton biomass (mg m⁻³), (d) cladoceran biomass (mg m⁻³), (e) copepod biomass (mg m⁻³), and (f) body size (μ m) by treatment and week of experiment. Error bars represent ± 1 SE.

3.3. Zooplankton methylmercury and total mercury concentrations

Zooplankton MeHg ranged from a low of 50.2 ng g⁻¹ dry weight (DW) to a maximum of 266.0 ng g⁻¹ DW across treatments, with an overall average of 136.2 ng g⁻¹ DW. Warming and nutrient treatments had a significant interactive effect on zooplankton methylmercury concentrations: at low temperatures, nutrients had a marginal positive effect on MeHg, whereas in the absence of nutrients zooplankton MeHg concentrations were higher in the temperature treatments than the controls across all dates (Figs. 3, 4, Table 4). Additionally, in the temperature treatment the addition of nutrients

reduced MeHg zooplankton concentrations compared to no nutrients (Fig. 4). In the no-nutrient treatment, zooplankton MeHg concentrations were 1.3 \times higher in the high temperature versus the low temperature treatment, but with the addition of nutrients, MeHg was reduced by 21% in the high compared to the low temperature treatment (Fig. 4). The largest effect was observed in the high temperature treatment, where the addition of nutrients reduced zooplankton MeHg by 31% compared to no nutrients (Fig. 4). No significant effects of treatments on total mercury (THg) concentrations were found at the end of the experiment (Fig. 3, Table 4) (due to low zooplankton mass in treatment mesocosms, THg was only analyzed

Table 2

Statistical summary of RM-ANOVA on zooplankton community data of abundance, biomass, and abundance-weighted body size for weeks 2–6 (i.e., day 7–35). Subscripts indicate degrees of freedom for RM-ANOVA. † $p < 0.1$; * $p < 0.05$.

Treatment	Abundance		Biomass		Body size	
	F-ratio	p-Value	F-ratio	p-Value	F-ratio	p-Value
Nutrient _[1,12]	4.566	0.054†	1.225	0.290	1.754	0.210
Temp _[1,12]	4.157	0.064†	2.512	0.139	0.010	0.921
Nutrient × Temp _[1,12]	0.251	0.626	0.026	0.875	1.742	0.211
Time _[4,48]	0.882	0.482	0.568	0.687	4.099	0.025*
Time × Nutrient _[4,48]	1.493	0.219	1.565	0.199	1.492	0.220
Time × Temp _[4,48]	0.299	0.877	0.909	0.466	0.210	0.932
Time × Nutrient × Temp _[4,48]	1.586	0.193	0.364	0.833	0.748	0.564

for Day 35). Zooplankton THg concentrations ranged from 103.0 to 276.6 ng g⁻¹ DW, averaging 191.3 ng g⁻¹ across treatments.

Zooplankton MeHg concentrations appeared to decline significantly with temperature ($R^2 = 0.164$, $p = 0.024$), though this affect may be related to sampling period, with a cluster of points from Day 14 separated from points on Day 35 (Fig. 5a,b). This may be the result of our study design, as the absence of a renewable source of MeHg is likely responsible for the overall decline in zooplankton MeHg over time (Fig. 3). When the different days are analyzed separately, we find that there is no relationship between temperature and zooplankton MeHg (Table 5). Given these temporal trends, we analyzed all relationships between predictor variables and zooplankton MeHg concentrations separately by day (Table 5). Notably, both total and cladoceran biomass were positively related to zooplankton MeHg on Day 14 at $\alpha = 0.05$, in addition to a weak positive relationship ($\alpha = 0.10$) between copepod biomass and zooplankton MeHg on Day 14 (Figs. 5, 6). There was a weak negative relationship ($\alpha = 0.10$) between edible chl *a* and zooplankton MeHg on Day 35 (Fig. 5, Table 5).

4. Discussion

Anthropogenic stressors, like climate change and excess nutrients, in freshwater systems can alter patterns of contaminant exposure and risk, necessitating a better understanding of interactive effects of environmental stressors. We aimed to determine if water temperature and nutrients altered zooplankton communities and phytoplankton biomass, and thus in turn impact zooplankton MeHg concentrations (Appendix A). The key findings from this study are that temperature increased MeHg concentrations in zooplankton compared to controls, but that nutrients appeared to mediate the effect of temperature on zooplankton MeHg (Fig. 4). However, there was high variability among mesocosms in this treatment, which we discuss below. We found that temperature had little, if weak, effects on overall phytoplankton and zooplankton biomass, but did interact with the nutrient treatment to alter community composition. Nutrients were associated with an increase in

Table 3

Statistical summary of RM-ANOVA on cladoceran and copepod metrics for weeks 2–6 (i.e., day 7–35). Subscripts indicate degrees of freedom for RM-ANOVA. † $p < 0.1$; * $p < 0.05$.

Treatment	Cladoceran: copepod biomass		Cladoceran biomass		Copepod biomass	
	F-ratio	p-Value	F-ratio	p-Value	F-ratio	p-Value
Nutrient _[1,12]	0.569	0.465	0.580	0.461	0.459	0.511
Temp _[1,12]	2.718	0.125	0.019	0.893	0.265	0.616
Nutrient × Temp _[1,12]	0.000	0.997	0.565	0.467	0.176	0.682
Time _[4,48]	1.380	0.255	0.587	0.673	1.760	0.152
Time × Nutrient _[4,48]	1.810	0.142	0.871	0.488	1.091	0.372
Time × Temp _[4,48]	0.614	0.655	0.508	0.730	0.535	0.710
Time × Nutrient × Temp _[4,48]	3.031	0.026*	0.649	0.630	0.823	0.517

phytoplankton over time, as was expected, with some indirect effects on zooplankton (i.e., increased copepod and total zooplankton biomass). 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 (see results-based model: Appendix A).

The temperature treatment appeared to increase zooplankton MeHg, resulting in some of the highest observed concentrations (Fig. 3). This result seems not to be the direct effect of higher temperatures on zooplankton MeHg (Fig. 5), but rather related to indirect effects of temperature on phytoplankton and zooplankton. However, when nutrients were added, the positive effect of temperature on MeHg was reversed, with the nutrient × temperature treatment having, on average, the lowest zooplankton MeHg concentrations. One possible mechanism for the increase in zooplankton MeHg in high temperature mesocosms is that elevated temperatures can increase feeding rates, and therefore, increase accumulation of contaminants (Dijkstra et al., 2013). We found that *Daphnia* were 1.3× more abundant in the high temperature treatment in the absence of nutrients compared to controls (i.e., significant nutrient × temperature interaction; Appendix C). Larger-bodied, less selective filter-feeders, such as *Daphnia*, can fare better in warmer systems, as they 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 established that warmer temperatures can result in higher filtering rates, especially in *Daphnia* (Burns, 1969). Thus, there is support for our prediction that increased feeding rates may have resulted in higher zooplankton MeHg.

We found that an increase in nutrients appeared to buffer zooplankton MeHg concentrations in the presence of warmer temperatures (Fig. 4). This result is consistent with other studies done in closed systems that have observed a negative correlation between phytoplankton biomass and zooplankton MeHg bioaccumulation (Pickhardt et al., 2002; Chen and Folt, 2005; Chen et al., 2005). However, it is worth noting that the nutrient × temperature treatment had the greatest variation (Fig. 3), whereby two mesocosms had low MeHg and two mesocosms had MeHg levels similar to those in the temperature treatment. The low MeHg mesocosms do not appear to be erroneous, as they were consistently low in all mercury measurements and were significantly correlated across dates (Day 14 and 35 MeHg: $r = 0.640$, $p = 0.014$) and mercury forms (Day 35 MeHg and THg: $r = 0.758$, $p = 0.001$). Rather, the two low MeHg mesocosms appear to be the result of some early differences in individual mesocosm responses to treatments: mesocosms with high MeHg had 33× more *Daphnia* than the low MeHg mesocosms on Day 14 and 2.5× more edible chl *a* on Day 7 (M. Jordan, unpublished). Indeed, we found a significant positive relationship between cladoceran biomass and zooplankton MeHg on Day 14 (Fig. 6a), similar to trends found in California reservoirs (Stewart et al., 2008). 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). These results suggest that our understanding of the accumulation of methylmercury in food webs will be incomplete without consideration of community composition.

Altogether, our findings suggest that nutrients may not actually moderate the effects of temperature on zooplankton MeHg. Instead, the combined effects of temperature (more of the efficient grazing *Daphnia*) and nutrients (more edible phytoplankton) may exacerbate MeHg accumulation in zooplankton if aqueous MeHg is not limiting. This result has important implications: given the projected increases in temperature related to climate change in freshwater systems, the addition of nutrients may not be able to ameliorate the temperature-related increase in zooplankton MeHg.

We predicted that nutrients would increase phytoplankton and therefore dilute concentrations of MeHg in zooplankton. Though the nutrient treatment consisted of a single pulse of added nutrients, the

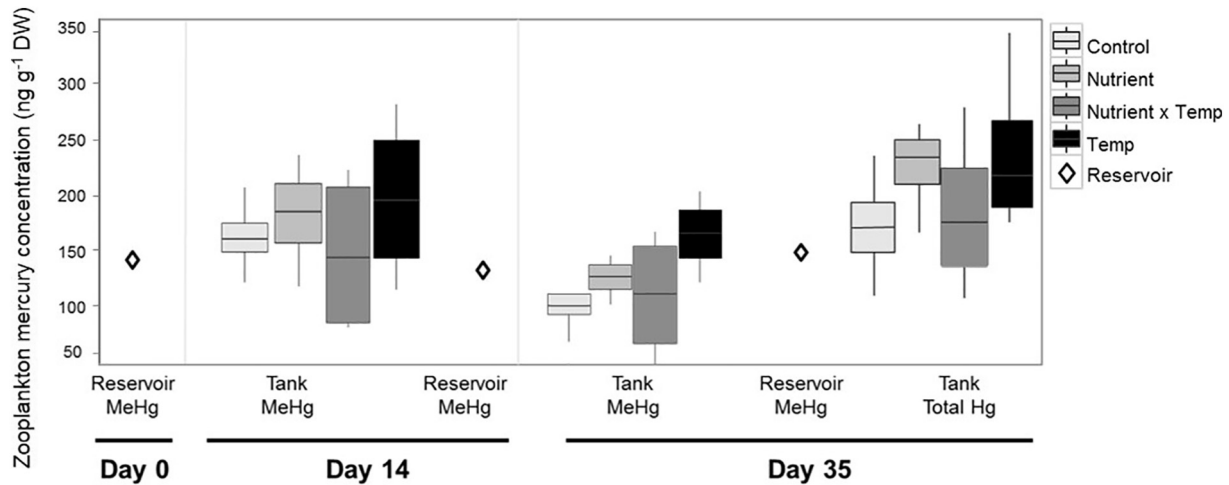


Fig. 3. Box and whisker plots of methylmercury and total mercury in zooplankton (ng g^{-1} , DW [dry weight]). Box represents interquartile range of values, with horizontal line as the median; whiskers represent minimum and maximum values. Single values represent single measurements from the reservoir zooplankton; on Day 0, reservoir zooplankton MeHg values are considered representative of mesocosm MeHg concentrations. Methylmercury in mesocosm zooplankton was measured on Day 14 and on Day 35 of the experiment, while total mercury was only measured on Day 35.

effect was significant enough to elevate edible chl *a* concentrations, especially on Day 7, where concentrations were almost 3× higher in the nutrient treatment compared to the no nutrient treatment (Fig. 1). We predicted that an increase in edible phytoplankton would result in biodilution and a reduction of MeHg concentrations of phytoplankton in the nutrient alone treatment, thus resulting in lower MeHg zooplankton concentrations, but it did not. Indeed, we saw slightly higher MeHg in the zooplankton from the nutrient treatment compared to controls (Fig. 3). We failed to see a significant direct relationship between either edible chl *a* and MeHg (Fig. 5c,d) or total chl *a* and MeHg (Day 14: $R^2 = 0.254$, $p = 0.055$; Day 35: $R^2 = 0.002$, $p = 0.871$), though some of these relationships were weakly negative, as is expected with biodilution. It is possible that this was due to the timing of the sampling for MeHg (Day 14 and 35) being out of sync with changes in phytoplankton, i.e., time lag effects. However, there was also no relationship between MeHg and either edible or total chl *a* from the previous week (Day 14 edible: $R^2 = 0.016$, $p = 0.657$; Day 14 total: $R^2 = 0.041$, $p = 0.470$; Day 35 edible: $R^2 = 0.170$, $p = 0.127$; Day 35 total: $R^2 = 0.208$, $p = 0.088$). An

alternative explanation is that changes in the phytoplankton community precipitated changes in zooplankton community composition, which subsequently affected MeHg concentrations. This seems plausible, as there were positive correlations between % edible chl *a* with cladoceran and total biomass, as well as edible and total chl *a* with copepod biomass. Kainz and Mazumder (2005) suggested that accumulation of MeHg in zooplankton was the result of the combined effects of the quantity of algae ingested and how much algae are retained by zooplankton, which could provide support for the increased zooplankton MeHg observed in nutrient treatments compared to controls. The concentrations of MeHg in water in Cottage Grove Reservoir were relatively high (unfiltered mean = 0.10 ng L^{-1} , filtered mean = 0.08 ng L^{-1} ; Eckley et al., 2015), suggesting that there is ample MeHg available for algal uptake in this system. Thus, our results suggest that the role of phytoplankton in mercury bioaccumulation in our study was likely more related to indirect changes in the zooplankton community as opposed to a direct biodilution effect, as observed in other studies (e.g., Watras and Bloom, 1992; Kuwabara et al., 2005; Stewart et al., 2008).

Species-level differences in phytoplankton could possibly account for some of the variance in zooplankton MeHg concentrations. Some species of algae like *Chlorella* have been found to be “hyper-accumulators” 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). Though we did not evaluate phytoplankton community composition,

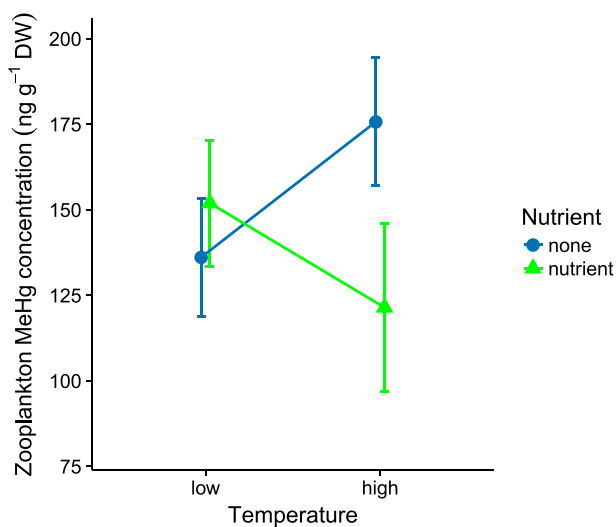


Fig. 4. Interaction plot of averaged methylmercury concentrations in zooplankton (ng g^{-1} , dry weight) over both mid- and end-points of experiment, as influenced by temperature and nutrients. Error bars represent ± 1 SE.

Table 4

Statistical summary of RM-ANOVA on zooplankton methylmercury (MeHg) and total mercury (THg). Subscripts indicate degrees of freedom. † $p < 0.1$; * $p < 0.05$.

Variable	Treatment	F-ratio	p-value
Zooplankton MeHg	Nutrient _(1,12)	3.787	0.065†
	Temp _(1,12)	0.000	0.994
	Nutrient × Temp _(1,12)	5.342	0.031*
	Time _(1,22)	3.717	0.067†
	Time × Nutrient _(1,22)	0.000	0.989
	Time × Temp _(1,22)	1.154	0.294
Zooplankton THg	Time × Nutrient × Temp _(1,22)	0.022	0.883
	Nutrient _(1,11)	0.037	0.850
	Temp _(1,11)	0.492	0.498
	Nutrient × Temp _(1,11)	1.590	0.233

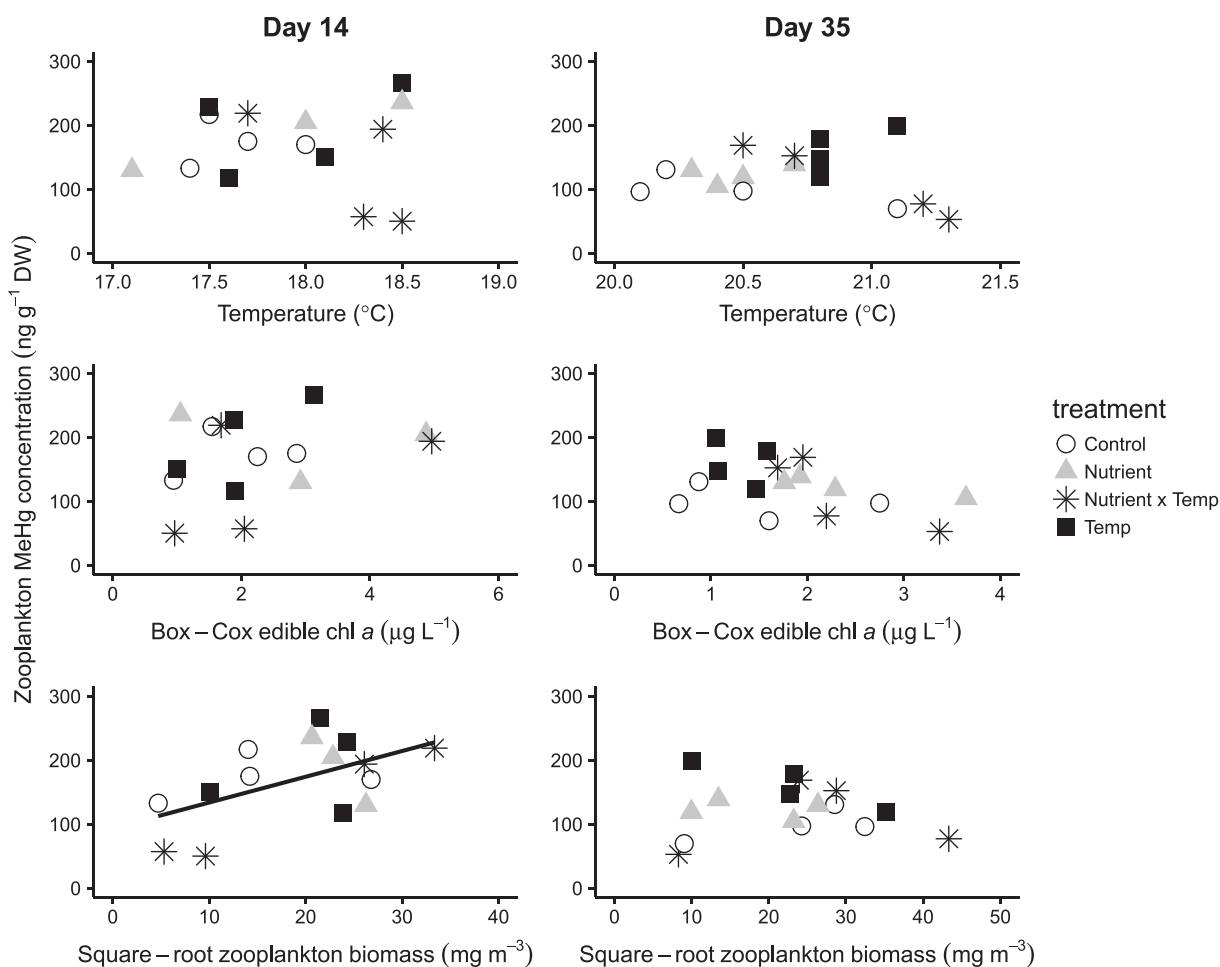


Fig. 5. Regressions of average zooplankton MeHg concentrations (ng g^{-1}) as a function of (a,b) temperature ($^{\circ}\text{C}$), (c,d) edible chl a ($\mu\text{g L}^{-1}$, Box-Cox transformation), and (e,f) zooplankton biomass (mg m^{-3} , square-root transformation) from Day 14 (left, $n = 15$) and Day 35 (right, $n = 16$). Regression for (e) zooplankton biomass and MeHg on Day 14: $y = 4.026x + 93.739$.

we found no treatment differences on the percent of total chlorophyll a that was composed of edible taxa, suggesting that there were no size-based shifts in algal communities (M. Jordan, unpublished). The relationship between algal community composition and methylmercury remains to be examined.

As with any mesocosm experiment, there are caveats to our study. We observed increased pH and dissolved oxygen over the course of the experiment, both of which can affect organismal biology and mercury accumulation (Morel et al., 1998). Warmer air temperatures in summer may result in higher primary productivity, which can increase pH and dissolved oxygen (Wetzel, 2001). Importantly, neither of these variables were substantially affected by the treatments (Appendix B). Periphyton on the insides of mesocosms was qualitatively observed to increase by experiment end, and this may have had some bearing on

the lack of temperature effect on chlorophyll a as free-floating phytoplankton. The reduction in MeHg over time (Fig. 3) may have been the result of photodemethylation, where MeHg is converted back to elemental Hg through UV radiation; the elemental Hg then volatilizes out of the system (Lehnerr and St. Louis, 2009). The presence of UV inhibitors in the greenhouse sheeting may have minimized methylmercury loss via photodemethylation. Other pathways, including bacterial demethylation, could have also contributed to MeHg losses (Seller et al., 1996; Marvin-DiPasquale et al., 2000). Given that we did not use sediments in our experiment, there was no opportunity for the Hg to cycle back into MeHg in the mesocosms and therefore, the effects that we observed were the result of existing aqueous MeHg. The absence of sediment could be important, as Luengen and Flegel (2009) found that there were significant increases in dissolved MeHg after an algal bloom, which they associated with the interaction of decaying phytoplankton and suboxic conditions in the surface sediments. These factors limited the existing MeHg in mesocosms over time. Because of this reduction, it is possible that potential treatment effects were influenced by overall loss of MeHg in these simulated systems, thus our results are likely conservative.

Even with these diminished MeHg concentrations, we were able to observe an interactive effect of temperature and nutrients on MeHg concentrations, which may be related to the availability of edible algae and the abundance of *Daphnia*. 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

Table 5
Regression models of predictor variables with zooplankton MeHg concentration on Day 14 and 35. † $p < 0.1$; * $p < 0.05$.

Predictor variable	Day 14		Day 35	
	R^2	p	R^2	p
Temperature	0.000	0.963	0.028	0.538
Edible chlorophyll a	0.080	0.306	0.216	0.070†
Zooplankton biomass	0.301	0.034*	0.002	0.866
Cladoceran biomass	0.315	0.029*	0.013	0.672
Copepod biomass	0.214	0.082†	0.075	0.303
Cladoceran:copepod	0.162	0.137	0.083	0.279
Average body size	0.093	0.268	0.022	0.585

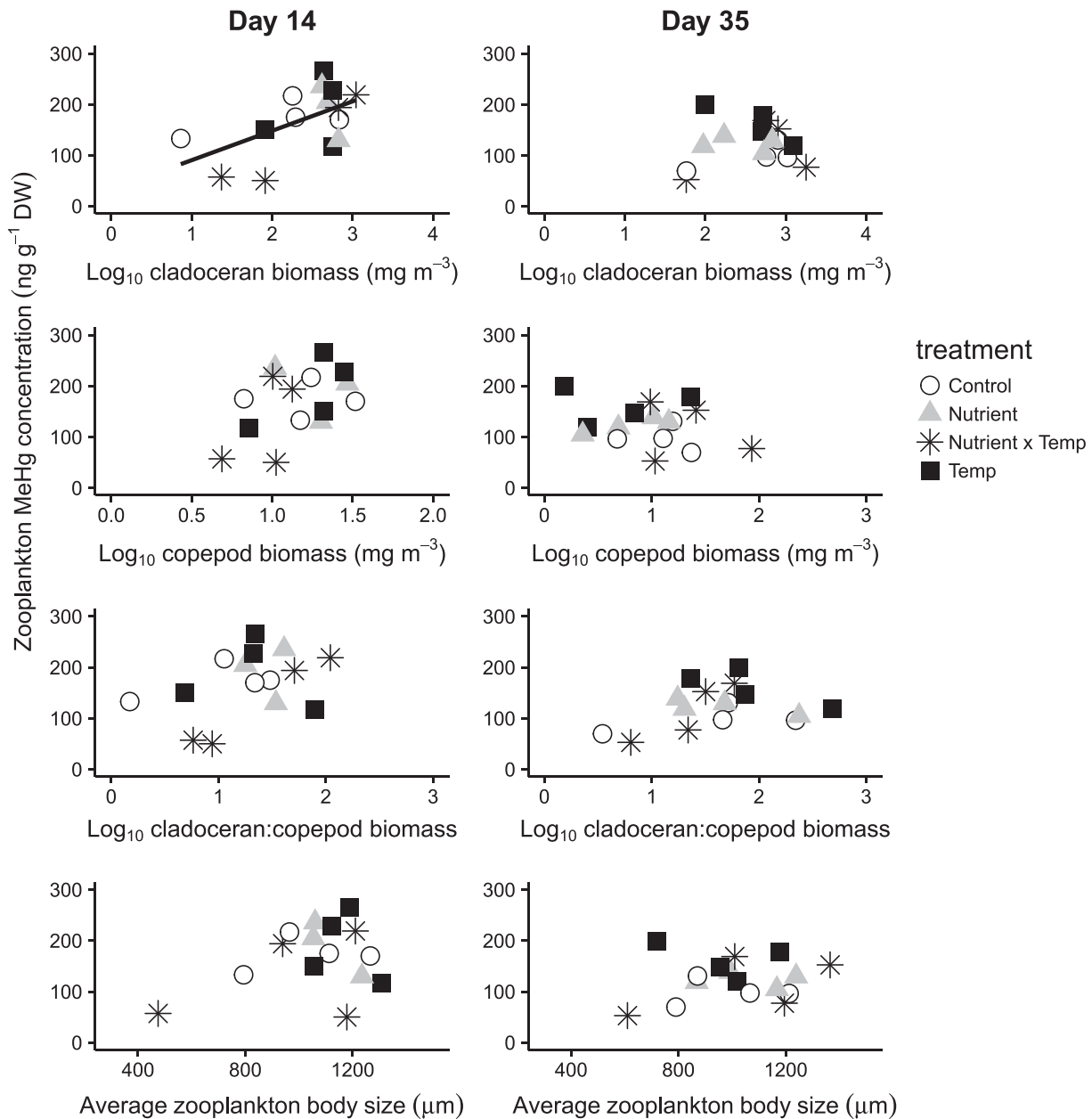


Fig. 6. Zooplankton MeHg concentrations (ng g^{-1}) as a function of (a,b) \log_{10} cladoceran biomass (mg m^{-3}), (c,d) \log_{10} copepod biomass (mg m^{-3}), (e,f) \log_{10} cladoceran:copepod biomass, and (g,h) zooplankton body size (μm) from Day 14 (left, $n = 15$) and Day 35 (right, $n = 16$). Regression for (a) cladoceran biomass and MeHg on Day 14: $y = 57.92x + 32.46$.

like Arctic ecosystems (Stern et al., 2012). We observed zooplankton MeHg concentrations that averaged 136.2 ng g^{-1} across treatments, which is relatively high compared to other studies in lakes and reservoirs (Kainz and Mazumder, 2005; Stewart et al., 2008; but see Kuwabara et al., 2006). Using reservoir water samples collected in the same summer by Eckley et al. (2015), we can estimate a bioaccumulation factor (BAF) for zooplankton, where:

$$\log_{10} \text{BAF} = \frac{\text{zooplankton MeHg (ng g}^{-1} \text{ DW)}}{\text{unfiltered water MeHg (ng L}^{-1})/1000} \quad (1)$$

Using Eq. (1), we observed an average \log_{10} BAF of 6.17 in our experimental mesocosms, which is very similar to the \log_{10} BAF of the reservoir (6.15). These values appear to be slightly higher compared to other studies (e.g., Stewart et al., 2008; Yu et al., 2011), suggesting that MeHg is transferred to zooplankton very efficiently in this system, which could

explain the high concentration of MeHg in top predators of Cottage Grove Reservoir (Curtis et al., 2013). The most recent data from Cottage Grove Reservoir (2003) found that piscivores ($>120 \text{ mm}$) averaged 1.63 mg kg^{-1} THg dry mass (Hope and Rubin, 2005), which represents a theoretical increase of over 1000%, 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. Gaining a better understanding of what might mitigate or amplify the harmful effects of MeHg is critical to present and future generations of people reliant on fisheries for recreation and consumption.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2019.02.259>.

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