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Methylmercury enters an aquatic food web through acidophilic microbial mats in Yellowstone National Park, Wyoming

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Summary

Microbial mats are a visible and abundant life form inhabiting the extreme environments in Yellowstone National Park (YNP), WY, USA. Little is known of their role in food webs that exist in the Park's geothermal habitats. Eukaryotic green algae associated with a phototrophic green/purple *Zygogonium* microbial mat community that inhabits low-temperature regions of acidic (pH ~ 3.0) thermal springs were found to serve as a food source for stratiomyid (Diptera: Stratiomyidae) larvae. Mercury in spring source water was taken up and concentrated by the mat biomass. Monomethylmercury compounds ($MeHg^+$), while undetectable or near the detection limit (0.025 ng l^{-1}) in the source water of the springs, was present at concentrations of 4–7 ng g^{-1} dry weight of mat biomass. Detection of $MeHg^+$ in tracheal tissue of larvae grazing the mat suggests that $MeHg^+$ enters this geothermal food web through the phototrophic microbial mat community. The concentration of $MeHg^+$ was two to five times higher in larval tissue than mat biomass indicating $MeHg^+$ biomagnification occurred between primary producer and primary consumer trophic levels. The *Zygogonium* mat community and stratiomyid larvae may also play a role in the transfer of $MeHg^+$ to species in the food web whose range extends beyond a particular geothermal feature of YNP.

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Introduction

Mercury is neurotoxic and can be very harmful to the central nervous system (Wiener *et al*., 2002). Mercury (Hg) is the only metal known to increase in concentration through all trophic levels of the aquatic food chain. A consequence of this phenomenon was the poisoning of many humans by methylmercury after eating fish that were highly contaminated by mercury from direct industrial sources (Hamada and Osame, 1996). The methylation of Hg and subsequent exposure of biota to monomethylmercury compounds ($MeHg^+$) are greater in aquatic than terrestrial environments. Monomethylmercury compounds enter the base of food webs as a result of uptake by primary producers (Mason *et al*., 1996; Pickhardt and Fisher, 2007). Biomagnification occurs when consumers absorb $MeHg^+$ from their organic food source and then respire carbon faster than they excrete the metal (Watras *et al*., 1998). In aquatic invertebrates, $MeHg^+$ is much more readily assimilated and bioaccumulated than is inorganic Hg (Wiener *et al*., 2002). Our understanding of $MeHg^+$ biomagnification is based largely on information obtained from studies of food webs that have been exposed to Hg introduced through relatively recent (< 500 years) anthropogenic activities (Cleckner *et al*., 1998; Roulet *et al*., 2000; Žižek *et al*., 2007). Less is known about bioaccumulation of $MeHg^+$ in aquatic food webs that have evolved in the presence of naturally high levels of Hg derived from geological processes and pathways even though one-third of the total mercury in global fluxes is estimated to arise from natural emissions (Mason *et al*., 1994; Friske and Coker, 1995; Shilts and Coker, 1995; Rasmussen *et al*., 1998; Roulet *et al*., 1998).

The present Yellowstone ecosystem which encompasses Yellowstone National Park (YNP), WY, USA, evolved during three catastrophic volcanic eruptions; the

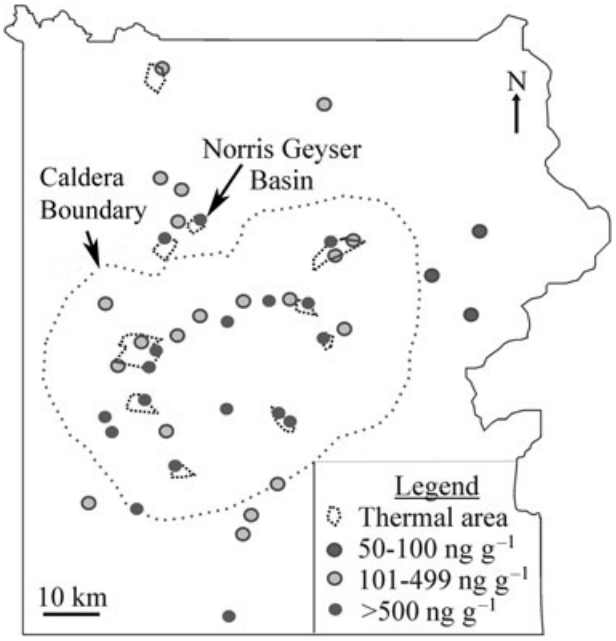

Fig. 1. Mercury levels in soils of Yellowstone National Park, WY, USA. Only major thermal areas are included. [Figure adapted from Phelps and Busick (1980).]

most recent occurred 642 000 years ago. Subsequent geothermal processes have produced what would normally be considered toxic concentrations of Hg in many geothermal soils and springs in YNP (Phelps and Buseck, 1980) (Fig. 1), many of which are also markedly acidic (pH = 1–3). Mats of *Zygogonium*, a green algae whose filaments often appear purple in colour due to a reaction between iron and tannins that accumulate in vacuoles within the cells, are common features of the acid thermal springs of YNP where temperatures are below 40°C. Components of the microbial community associated with *Zygogonium* mats are a food resource for aquatic insects (Brock *et al*., 1969; Collins, 1975). Studies in other freshwater and marine systems have shown that algae take up and concentrate both Hg(II) and $MeHg^+$ from their aqueous surroundings (Fisher *et al*., 1984; Mason *et al*., 1996; Miles *et al*., 2001). Recently, it was shown that phototrophic microbial assemblages attached to the substratum of several acidic springs of YNP accumulate total Hg (THg) and $MeHg^+$ (King *et al*., 2006). The purpose of this study is to investigate trophic interactions and accumulation of $MeHg^+$ in a YNP aquatic food web comprised of acidophilic and/or acid-tolerant species that likely evolved in the presence of naturally high concentrations of Hg from a geothermal source. The results of the study suggest that the *Zygogonium* microbial mat community serves as a point of entry of $MeHg^+$ into this YNP food web.

Results and discussion

Terminal-restriction fragment length polymorphism (T-RFLP) provided the first direct field evidence that phototrophic microbial populations associated with *Zygogonium* mats serve as a food source for stratiomyid (Diptera: Stratiomyidae) larvae taxonomically related to *Odontomyia occidentalis* (98.6% 28S rRNA gene sequence homology). The ribulose-1,5-bisphosphate carboxylase (*rbcL*) gene was selected as a phylogenetic marker for phototrophic microbial populations because the enzyme it encodes is involved in CO_2 fixation, a pathway that is not known to exist in aquatic insects. DNA recovered from the *Zygogonium* mat community of an acid-sulfate-chloride spring, informally referred to as Succession Spring in the Norris Geyser Basin of YNP (Macur *et al*., 2004), and DNA from the foregut of stratiomyid larvae associated with the mat contained common terminal-restriction fragments (T-RFs). Of the 9 and 13 different length T-RFs recovered from mat and stratiomyid foregut DNA, respectively, four were shared (Table 1). The four common phylotypes represented 43.1% and 63.1% of the total fluorescence of the

Table 1. Terminal restriction fragment profiles of PCR-amplified *rbcL* genes of microbial communities in stratiomyid larval foregut and *Zygogonium* mat.

	T-RF abundance as a percentage of total community fluorescence			
	Succession Spring[a]		Dragon Spring[a]	
T-RF (bp)[b]	Foregut	Mat	Foregut	Mat
148			4.1 (1.0)[c]	
161		9.5 (1.0)		6.3 (2.9)
220	3.3 (0.5)		3.6 (1.8)	
233	4.6 (1.2)		4.1 (3.7)	
239	3.5 (1.1)	5.8 (1.8)		
245	5.6 (0.7)		4.7 (3.4)	
263		13.0 (1.1)		6.0 (2.6)
391	7.6 (0.3)		7.5 (0.8)	
418	5.9 (1.2)	17.9 (2.1)		12.1 (3.3)
421	16.9 (2.2)		3.7 (1.0)	
424			3.5 (0.4)	
438		4.4 (0.9)		4.3 (1.9)
466		4.9 (1.4)		6.2 (5.2)
478			4.1 (0.9)	
529	4.4 (0.4)		4.4 (0.1)	
532	5.3 (0.3)		5.6 (0.2)	
535	5.7 (0.3)		6.1 (0.3)	
593	8.2 (1.0)	14.9 (3.5)	6.6 (0.3)	23.3 (9.1)
618	3.3 (0.9)		4.1 (1.8)	
645		5.1 (0.4)		11.6 (0.9)
668	25.5 (5.0)	24.5 (3.9)	31.4 (5.2)	22.5 (8.9)
693				3.9 (1.2)
738				3.7 (2.2)
Total	100.0	100.0	100.0	100.0

a. Samples collected on 2 June 2004.
b. Terminal restriction fragment (T-RF) as base-pair (bp) length.
c. Standard deviations of three replicate profiles reported in parentheses.

Table 2. Mercury in biotic and abiotic compartments of acidic thermal springs of Yellowstone National Park

Compartment	Site		
	Succession Spring	Dragon Spring	Hazle Lake Spring
THg in filtered spring water adjacent to mat (ng l^{-1})	94 ± 25,[a,b] 63[c]	38[b]	ND
THg in filtered interstitial water of mat (ng l^{-1})	71[c]	ND	2[d]
$MeHg^+$ in filtered spring water adjacent to mat (ng l^{-1})	< 0.025[b]	< 0.025[b]	ND
$MeHg^+$ in filtered interstitial water of mat (ng l^{-1})	1.62[c]	ND	0.03[d]
THg in mat biomass (ng g^{-1} dry weight)	18 000 ± 5300[e]	15 000 ± 4700[e]	157 ± 47[d]
$MeHg^+$ in mat biomass (ng g^{-1} dry weight)	5.8 ± 4.2[e]	7.3[e]	4.0 ± 1.2[d]
Mat THg Bf [log(ng kg^{-1} dry weight biomass) (ng l^{-1} interstitial water)$^{-1}$]	5.4	ND	4.9
Mat THg Bf [log(ng kg^{-1} dry weight biomass) (ng l^{-1} adjacent spring water)$^{-1}$]	5.5	5.6	ND
Mat $MeHg^+$ Bf [log (ng kg^{-1} dry weight biomass) (ng l^{-1} interstitial water)$^{-1}$]	3.6	ND	5.1
Mat $MeHg^+$ Bf [log (ng kg^{-1} dry weight biomass) (ng l^{-1} adjacent spring water)$^{-1}$]	> 5.4	> 5.5	ND
% THg as $MeHg^+$ in mat biomass [(ng $MeHg^+$ g^{-1} dry weight) (ng THg g^{-1} dry weight)100]	3.2×10^{-2}	4.9×10^{-2}	2.5
$MeHg^+$ in larvae whole body (ng g^{-1} wet weight)	1.6[e]	2.0[e]	1.2[d]
Larvae whole body $MeHg^+$ Bf [log (ng kg^{-1} wet weight biomass) (ng l^{-1} adjacent spring water)$^{-1}$]	> 4.8	> 4.9	4.6[f]
$MeHg^+$ in larval gut (ng g^{-1} dry weight)	11.4[e]	14.5[e]	1.9[d]
$MeHg^+$ in larval tracheal tissue (ng g^{-1} dry weight)	23.5[e]	33.7[e]	7.9[d]
$MeHg^+$ biomagnification [(ng g^{-1} dry weight larval tracheal tissue) (ng g^{-1} dry weight mat biomass) $^{-1}$]	4.1	4.7	2.0

a. ±1 SD, $n = 2$ when replicate samples were collected.
b. Sample collected on 18 April 2006.
c. Sample collected on 17 May 2006.
d. Sample collected on 22 September 2006.
e. Sample collected on 20 May 2005.
f. Based on $MeHg^+$ concentration in mat interstitial water.
ND, no data.

larval foregut and mat community T-RFLP profiles respectively. The recovery of the four T-RFs (phylotypes) from both the foregut of larvae and the mat community suggests selective grazing of these mat phylotypes by the stratiomyid larvae.

A similar stratiomyid grazing pattern was observed in Dragon Spring, another acid-sulfate-chloride spring in Norris Geyser Basin (D'Imperio *et al*., 2007). Of the 10 and 14 different length T-RFs recovered from mat and stratiomyid foregut DNA, respectively, two were shared (Table 1). The two common phylotypes contributed 38.0% and 45.8% of the total fluorescence of the larval foregut and mat communities of this spring respectively. Both phylotypes common to the mat and foregut of larvae collected from Dragon Spring were among the four phylotypes common to the larval foregut and *Zygogonium* mat samples from Succession Spring. These results suggest that stratiomyid larvae graze similar mat-associated phylotypes in geographically distinct springs.

The taxonomy of grazed *Zygogonium* mat phylotypes was determined by sequencing cloned *rbcL* gene amplicons that yielded a T-RF corresponding in size to those that were shared by mat and larval foregut communities. Analysis of the translated sequence revealed that the shared 668-base-pair (bp) phylotype was most closely affiliated with *Leptosira terrestris* (97% similarity), whereas the shared 593 bp phylotype was most closely affiliated with a *Chlorella* sp. (95% similarity), both of which are eukaryotic green algae within the phylum *Chlorophyta*. Transformants were not recovered from the library that produced a T-RF corresponding in size to the 239 or 418 bp shared phylotypes. These results suggest that eukaryotic green algae associated with the *Zygogonium* mat are a food resource for stratiomyid larvae that inhabit these springs.

Filtered spring water adjacent to the *Zygogonium* mat grazed by the stratiomyid larvae of Succession and Dragon Springs contained THg concentrations > 29 ng l^{-1} (Table 2), well above the 2–4 ng l^{-1} measured in water in YNP devoid of thermal inputs (D.K. Nordstrom, unpubl. data). Dissolved $MeHg^+$ was undetectable (0.025 ng l^{-1} detection limit) in the water adjacent to the *Zygogonium* mats of Succession and Dragon Springs (Table 2). The results were consistent with those from all but one other acidic spring (Frying Pan West Spring; Table S1) sampled in this study, and with results of other studies on acidic springs in YNP that contained high concentrations of THg in the filtered bulk aqueous phase (Frying Pan East Spring and Roadside East Spring; Table S1). The THg concentration (71 ng l^{-1}) in a single

sample of interstitial water recovered from the *Zygogonium* mat of Succession Spring was similar to that (63 ng l^{-1}) in a sample of spring water adjacent to the mat collected on the same date (Table 2). In contrast, the $MeHg^+$ concentration (1.62 ng l^{-1}) in a single sample of *Zygogonium* mat interstitial water was >65 times that measured in the adjacent spring water 1 month earlier (Table 2). Collection of replicate interstitial water samples was not allowed under the YNP research permit (Permit No. YELL-2006-SCI-5134) because of the disruptive influence sampling has on mat structure and integrity. Nevertheless, this small data set suggests that the *Zygogonium* mat or underlying sediment in Succession Spring may be a site of Hg methylation, as demonstrated previously for sessile microbial assemblages in other aquatic systems (Cleckner *et al*., 1999; Guimaraes *et al*., 2000; Desrosiers *et al*., 2006), and more recently in acidic mine tailings at a pH similar to that of Succession Spring (Winch *et al*., 2008). Although establishment of a role for the mat microbial community in Hg(II) methylation was beyond the scope of this study, preliminary results of a recent radioisotope experiment revealed that the rate of methylation of $^{203}HgCl_2$ by samples of mat from Succession Spring incubated in the absence of glutaraldehyde was significantly greater than in the presence of this inhibitor of biological activity (G. Geesey and T. Barkay, unpubl. results).

There are acidic springs in YNP with a geothermal source which contain concentrations of THg that are comparable to the lowest concentrations measured in springs devoid of thermal inputs. Hazle Lake Spring is one such spring that also supports *Zygogonium* mats grazed by stratiomyid larvae. Correspondingly, THg and $MeHg^+$ concentrations in the interstitial water of Hazle Lake Spring mats were only 2 and 0.03 ng l^{-1} respectively (Table 2). This provided the opportunity to compare Hg accumulation in mat and larval biomass in springs containing high and low concentrations of Hg in the spring water.

The THg concentrations in *Zygogonium* mat biomass in Succession and Dragon Springs were significantly higher ($P = 0.04$ and 0.05, respectively, two-tailed *t*-test) than in *Zygogonium* mat biomass of Hazle Lake Spring (Table 2). The results are consistent with those of other studies in which THg in primary producer biomass varies directly with the concentration of THg in the water (Žižek *et al*., 2007). In contrast, the concentration of $MeHg^+$ in mat biomass of Hazle Lake Spring was not significantly different ($P = 0.61$) from that in mat biomass of Succession Spring (Table 2). These results are consistent with those of other studies which suggest little or no relationship between the concentration of $MeHg^+$ in primary producer biomass and THg concentration in the water (Cleckner *et al*., 1998; Žižek *et al*., 2007). Dragon Spring was not included in this comparison because the reported concentration for $MeHg^+$ in mat biomass was obtained from a single sample.

The extent to which aquatic organisms accumulate Hg from the surrounding aqueous phase is often expressed as the bioconcentration factor (Bf), where $Bf = \log(C_b/C_w)$, and C_b and C_w are Hg concentrations in biota and water respectively (Watras and Bloom, 1992). Although Bf is typically calculated using biomass wet weight estimated from dry weight measurements and assumptions of biomass water content (Watras and Bloom, 1992; Hill *et al*., 1996; Watras *et al*., 1998), the highly variable water content of *Zygogonium* mat material prompted the substitution of dry weight for wet weight values for Bf calculations in the present study. Positive Bf values were obtained from the concentrations of THg and $MeHg^+$ in mat biomass and mat interstitial water from Succession and Hazle Lake Springs (Table 2). Similar Bf values were obtained when THg and $MeHg^+$ concentrations in spring water adjacent to the mat in Succession Spring were substituted in the Bf calculation for THg and $MeHg^+$ concentrations in the interstitial water (Table 2). These results indicate that the mat biomass concentrated these forms of Hg from the surrounding aqueous phase. Bioconcentration of THg and $MeHg^+$ in biomass of sessile microbial assemblages appears to be common in acidic springs containing high THg concentrations in other areas of YNP (e.g. green and purple-green mats of Frying Pan Bowl Spring and Frying Pan West Spring, and non-photosynthetic pink-tan streamers of Frying Pan East Spring, Frying Pan West Spring and Roadside East Spring) (Table S1).

While a number of studies have investigated the relationship between THg and $MeHg^+$ concentrations in natural waters and aquatic sediments (Gilmour and Henry, 1991; Watras *et al*., 1995; Benoit *et al*., 1998), few have examined this relationship in biomass of phototrophic sessile aquatic microbial assemblages. No strong correlation was observed between the concentrations or log concentrations of THg and $MeHg^+$ in biomass of phototrophic sessile microbial assemblages in acidic springs of YNP (Fig. 2, inset; $R^2 = 0.46$ and < 0.01 respectively), nor in biomass of periphyton in other aquatic systems (Fig. 2, inset; $R^2 = 0.06$ and 0.56 respectively) that contained high (> 29 ng l^{-1}) concentrations of THg in the aqueous phase.

The concentration of $MeHg^+$ relative to THg in YNP *Zygogonium* mat biomass appeared to depend on the concentration of THg in the spring water. The percentage of THg as $MeHg^+$ in biomass of mats from springs with high THg concentrations in the filtered aqueous phase (Succession and Dragon) was several orders of magnitude less than that for biomass of mat in Hazle Lake Spring, which contained a low concentration of THg in the filtered aqueous phase (Fig. 2; Table 2). A similar pattern

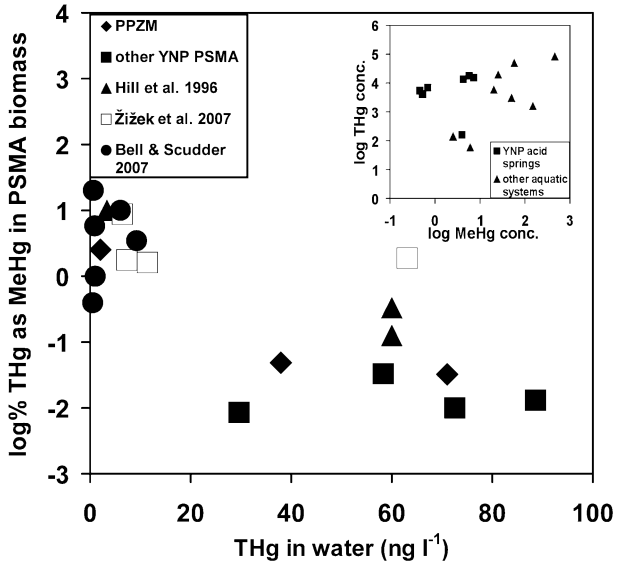

Fig. 2. Percentage of THg as $MeHg^+$ in biomass of photosynthetic sessile microbial assemblages (PSMA) [(ng $MeHg^+$ g^{-1} dry weight biomass)(ng THg g^{-1} dry weight biomass)$^{-1}$ 100] versus THg concentration in the filtered aqueous phase sample for various aquatic systems. PPZM, purple phototrophic *Zygogonium* mat.

was observed for periphyton in some (Hill *et al*., 1996) (Fig. 2; Table S1) but not all (Žižek *et al*., 2007) (Fig. 2; Table S1) Hg-contaminated lotic environments.

Sessile microbial assemblages in acidic springs of YNP containing high aqueous phase THg concentrations had lower percentages of THg as $MeHg^+$ than sessile microbial assemblages from other Hg-contaminated aquatic systems investigated to date (Hill *et al*., 1996; Bell and Scudder 2007; Žižek *et al*., 2007) (Fig. 2; Table S1). At sites containing high (> 29 ng l^{-1}) THg concentrations in the aqueous phase, the low percentage appeared to be more the result of the low concentration of $MeHg^+$ as opposed to a high concentration of THg in *Zygogonium* mat biomass. Methylmercury compounds in the acidic waters of YNP may exist in a form that is less bioavailable to the sessile microbial assemblages than those that exist in other aquatic systems. Furthermore, the sessile microbial assemblages in YNP acidic springs containing high THg concentrations may possess mechanisms to minimize $MeHg^+$ accumulation that have not yet evolved in sessile microbial assemblages in aquatic systems exposed to elevated concentrations of THg introduced through more recent human activities. A low concentration of $MeHg^+$ in biomass of phototrophic microbial mat communities exposed to high concentrations of aqueous phase THg may temper its biomagnification through the food web of these acidic springs.

The concentration of $MeHg^+$ in whole body extracts was similar for stratiomyid larvae recovered from Succession, Dragon and Hazle Lake Springs (Table 2). These concentrations are not likely to be toxic as they are below the 20 ng g^{-1} wet weight concentration that is considered to be a background concentration in amphibians and other aquatic organisms (Zoll *et al*., 1988). Methylmercury Bf values for larvae calculated from concentrations based on whole body wet weight and concentrations in filtered spring water adjacent to the mats in Succession and Dragon Springs or in filtered interstitial water of mats in Hazle Lake Spring were also similar for these three springs (Table 2). This reflects the fact that the $MeHg^+$ concentration in the filtered aqueous phase as well as that for whole body extracts of larvae was similar at these sites. The Bf values were also similar to those reported for aquatic insects at the same trophic level in other systems containing comparable concentrations of $MeHg^+$ in the aqueous phase. Bioconcentration factors of 4.0–4.3 were obtained from the $MeHg^+$ concentration in whole specimens of nymphs of the mayfly *Hexagenia* (Insecta: Ephemeropta) exposed to test water in contact with Hg-containing sediments from Whitehall Reservoir in the Sudbury River basin (MA, USA) (Naimo *et al*., 2000). The Bf values were also similar to that (4.3) calculated from a sample of pooled aquatic insects (collectors and shredders) and the surrounding water of an artificially flooded (1993 post flood) reservoir (L979) in the Experimental Lakes Area in north-western Ontario, Canada (Kelly *et al*., 1997; Hall *et al*., 1998).

While it has been shown that larvae of aquatic insects can accumulate $MeHg^+$ from sediment particles and dissolved and suspended particulate organic matter in the aqueous phase, it is generally assumed that they accumulate Hg(II) and $MeHg^+$ predominantly from their food (Odin *et al*., 1995; Tremblay and Lucotte, 1997; Sjoblom *et al*., 2000). Methylmercury compounds were detected in pooled gut and tracheal tissue from stratiomyid larval specimens collected from Succession, Dragon and Hazle Lake Springs (Table 2). The concentration of $MeHg^+$ in tracheal tissue was higher than the concentration in the gut from larval specimens from the three springs. This likely reflects the fact that $MeHg^+$ in the gut has not been fully digested and assimilated.

The concentration of $MeHg^+$ associated with the gut and tracheal tissue of larvae from Hazle Lake Spring was lower than that in the corresponding material from larvae of Succession and Dragon Springs (Table 2). Such a difference was not expected since the $MeHg^+$ concentrations in mat biomass from Succession Spring and Hazle Lake Spring were not significantly different (Table 2). If the larvae of Succession and Hazle Lake Springs grazed the same mat microbial populations as appears to be the case in Succession and Dragon Springs (Table 1), and mat biomass is the primary source of $MeHg^+$ that accumulates in the larval tissue, one would expect similar biomagnification values rather

than the more than twofold difference that was observed (Table 2). The need to pool tissue from the limited number of larval specimens permitted for collection from each spring into a single sample to obtain detectable quantities of $MeHg^+$ precluded a statistical analysis of these differences. An application has recently been submitted for a research permit to collect a sufficient number of larval specimens from each spring to determine whether the differences are significant.

Biomagnification resulting from the transfer of $MeHg^+$ from mat biomass to the larval tracheal tissue for Succession, Dragon and Hazle Lake Springs (Table 2) fell within the range (0.26–36) reported for aquatic insect larvae that graze other phototrophic microbial assemblages (Rask *et al*., 1994; Žižek *et al*., 2007) and within the range (1.6–35) reported for other aquatic primary consumers feeding on microbial primary producers (Cleckner *et al*., 1998; Herrin *et al*., 1998; Watras *et al*., 1998). The results provided no evidence that selective grazing of mat microbial populations attenuated bioaccumulation and biomagnification of $MeHg^+$ in larvae in the high-THg water of Succession and Dragon Springs, although linking these processes would be difficult to demonstrate *in situ*. Furthermore, there is no evidence to suggest that stratiomyid larvae in these high-THg geothermal systems were more efficient than primary consumers in other Hg-impacted environments in minimizing incorporation of food source-derived $MeHg^+$ in their tissues. The similarity in $MeHg^+$ biomagnification from mat biomass to larval tissue in the two springs suggests that $MeHg^+$ ingestion and assimilation by larvae was similar at these two sites (Table 2). Because the T-RFLP results indicated that the larvae in both springs grazed similar algal populations of the mat, it is likely that the $MeHg^+$ detected in the larval gut resulted from ingestion of this food source. Thus, the *Zygogonium* microbial mat community appears to serve as a point of entry for $MeHg^+$ into this geothermal food web. Further studies are needed, however, to determine the importance of the mat community relative to other potential routes of transfer of $MeHg^+$ to the stratiomyid larvae (primary consumer trophic level) in this geothermal food web.

Methylmercury has been shown to bioaccumulate in avian species as a result of predation on $MeHg^+$-containing invertebrates (Braune, 1987). Killdeer (*Charadrius vociferous*), which are known to graze ephydrid (Diptera: Ephydridae) (brine fly) larvae that inhabit phototrophic sessile microbial assemblages in springs of YNP (Brock, 1978), may bioaccumulate $MeHg^+$ by feeding on $MeHg^+$-containing stratiomyid larvae. During a sampling trip, a Killdeer was observed feeding on these larvae in Dragon Spring. A molted feather discarded by the bird was recovered and analysed for $MeHg^+$. Feather Hg levels are a useful matrix for evaluating chronic body burdens (Burger, 1993). The feather contained 202 ng g^{-1} dry weight $MeHg^+$, a sixfold enrichment relative to the levels in the larval tissue, and approaching the concentration (400 ng g^{-1} dry weight) accepted as threshold for protection of fish-eating birds (Mora *et al*., 2002). Based on this preliminary result, the *Zygogonium* microbial mat community may also play a role in the transport of $MeHg^+$ to species in the food web that range beyond this particular geothermal feature to other areas within and outside the boundaries of YNP. Larvae of stratiomyids, like those of ephydrids, may also serve as a food resource for predatory aquatic insects at the next trophic level. Ephydrid larvae, which also graze YNP phototrophic microbial mats, are the prey of mites and spiders (Brock, 1978). Thus, the *Zygogonium* mat-based food web through which $MeHg^+$ biomagnifies is likely to be more complex than described above.

In conclusion, stratiomyid larvae taxonomically related to *O. occidentalis* utilized, as a food source, eukaryotic green algae which were a dominant component of the phototrophic *Zygogonium* microbial mat community of acidic thermal springs in YNP. The biomass of these mats concentrated THg and $MeHg^+$ from the spring water. The percentage of THg as $MeHg^+$ in biomass of mats in springs containing high aqueous-phase THg concentrations was lower than that observed for periphyton in other aquatic systems containing comparable concentrations of THg in the aqueous phase. Methylmercury compounds were detected in the gut and tracheal tissue of larvae grazing the *Zygogonium* mats suggesting that this sessile microbial community serves as a point of entry of $MeHg^+$ into the geothermal food web. The microbial community may also play a role in the transfer of $MeHg^+$ to species in the food web whose range extends to other areas within and outside the boundaries of YNP.

Experimental procedures

Site description

Three springs, unofficially named Succession Spring (Northing 4952916, Easting 522734; NAD83 Grid 12), Dragon Spring (Northing 4953218, Easting 522889) and Hazle Lake Spring (Northing 4955312, Easting 522824), served as primary field sites for this study. Succession and Dragon Springs are located in Norris Geyser Basin, YNP, WY, USA. Both springs drain to the north, lack observable obstacles which might influence total radiance, have similar elevations (2289 and 2288 m for Succession and Dragon Springs respectively), and have separate source and drainage areas. Hazle Lake Spring is an acidic thermal spring within the boundaries of YNP in a watershed that extends along a north–south fault between Norris Geyser Basin and Mammoth Hot Springs. Locations and features of Frying Pan Bowl Spring and Frying Pan West Spring have been described elsewhere (King *et al*., 2006).

Identification of larvae

Larval specimens for taxonomic analysis were collected from Succession and Dragon Springs with sterile forceps (larval specimens), transferred to sterile 1.5 ml tubes, and placed on dry ice for transport back to the laboratory at Montana State University, Bozeman. Tracheal tissue (20 mg) was aseptically recovered from larval specimens and subjected to bead-beating nucleic acid extraction according to the manufacturer's directions (FastDNA Spin Kit for Soil, Q-Biogene, Irvine, CA). DNA concentration was determined using the High DNA Mass Ladder (Invitrogen, Carlsbad, CA) and 20 ng of larval DNA was used as template for PCR amplification of a 500 bp fragment of the 28S rDNA using primer pair rc28C and 28P (Wiegmann *et al*., 2000). PCR, cloning and sequencing were performed according to previously published protocols (Boyd *et al*., 2007). The sequence has been submitted to the DDBJ/EMBL/GenBank databases under the Accession No. DQ782815. Reference sequences used in analysis of the stratiomyid 28S rDNA sequence and construction of the phylogenetic tree shown in Fig. S1, and method of tree construction can be found in Appendix S1.

Terminal-restriction fragment length polymorphism (T-RFLP)

Terminal-restriction fragment length polymorphism was used to compare *rbcL* genes recovered from larval foregut and *Zygogonium* microbial mat. Specimens of larvae grazing the mat were collected from Succession and Dragon Springs as described above. Intact foregut was removed from the larvae recovered from each spring and distributed into sterile bead-beating tubes (three tubes per spring). Samples of mat biomass adjacent to the grazing larvae were collected with sterile syringes and distributed into sterile bead-beating tubes (three tubes per spring). The contents of each tube were subjected to bead-beating nucleic acid extraction and the DNA concentration was determined as described above. Extracted DNA (20 ng) was used as template for PCR amplification of the *rbcL* gene using primers 595F (5′-GACTTCACCAAAGACGACGA-3′) and 1387R (5′-TCGAACTTGATTTCTTTCCA-3′) (Elsaied and Naganuma, 2001) conjugated with FAM at the 5′-terminus (Invitrogen). Thirty-five cycles of PCR were performed in triplicate using an annealing temperature of 52°C. PCR products were purified using Wizard PCR Preps DNA Purification System (Promega) and the DNA concentration was determined as described above. Approximately 200 ng of PCR-amplified DNA from larval foregut and mat was subjected to digestion at 37°C for 12 h using the restriction enzyme MspI (C^CGG) (New England BioLabs, Ipswich, MA) as previously described (Boyd *et al*., 2007). A 50-fold excess of enzyme and 12-fold excess digestion time (based on an activity of 1 μg of DNA digested h^{-1} U^{-1} enzyme at 37°C) were used to ensure complete digestion of amplicons. Restriction digests were purified and analysed as previously described (Boyd *et al*., 2007).

Terminal-restriction fragments were analysed using Genescan version 2.1 (Applied Biosystems, Foster City, CA). Terminal-restriction fragments that migrated to within 1.0 bp of each other were considered to have originated from the same template. Only T-RFs present in all three replicate digests were retained for community characterization. Total fluorescence contributed by the sum of all T-RFs in each digest was standardized to a value of 10 000 units (Blackwood *et al*., 2003). Composite T-RF community profiles were generated from the triplicate digests by averaging peak migration distances and the per cent total fluorescence for each T-RF. According to previously established criteria (Boyd *et al*., 2007), a T-RF must contribute at least 2% of the total fluorescence in order to be included in a community. All T-RFs contributing $< 2.0\%$ total fluorescence were discarded without further consideration and the total community total fluorescence was again adjusted to 10 000 units.

Construction of rbcL *gene clone library and establishment of taxonomic affiliation of clones*

For the purposes of this study, unique T-RFs represent distinct operational taxonomic units, the unit by which individual phylotypes (populations) are demarcated. In order to assign taxonomic affiliation to individual phylotypes grazed by larvae, a clone library was constructed of *rbcL* gene amplicons generated from DNA extracted from the *Zygogonium* mat of Dragon Spring. PCR, amplicon purification and determination of DNA concentration were performed as described previously (Boyd *et al*., 2007). Primers used in sequencing were M13F and M13R. Processed sequences were aligned using CLUSTALW (http://clustalw.genome.jp/) and were grouped (100% sequence homology threshold) using the Sequence Grouper program (Andrew Shewmaker, Idaho National Laboratory, Idaho Falls, Idaho). A representative of each group was subjected to translated query-translated database (TBLASTX) analysis (Altschul *et al*., 1997) against the non-redundant database provided by the National Center for Biotechnology Information (NCBI).

A representative clone from each grouping was subjected to T-RF analysis in order to establish the taxonomic affiliation of each T-RF generated from the contents of the larval foregut and from the mat on which the larvae grazed. Purified plasmids were subjected to PCR amplification as described above for community amplifications for T-RFLP with primers 595F and 1387R-FAM using 20 ng of purified plasmid as template. PCR amplicons were purified, quantified and subjected to digestion as described above using 25 ng of DNA for each digest. Clone T-RF profiles were generated as described above for construction of community T-RF profiles. Clone T-RFs and T-RFs from foregut and mat samples were considered to be the same if they migrated to within 0.5 bp of each other. Clone *rbcL* gene sequences have been submitted to the DDBJ/EMBL/GenBank databases above under Accession Nos DQ398806–DQ398847.

Collection and processing of microbial mat, larvae and avian feather for Hg analysis

Samples of green and purple/green microbial mat from Frying Pan Bowl, Frying Pan West, Succession, Dragon and Hazle Lake Springs, as well as specimens of mat-associated stratiomyid larvae from Succession, Dragon and Hazle Lake Springs, and a single feather molted from a Killdeer (*C. vo-*

ciferous) grazing on mat-associated larvae in Dragon Spring were collected with acid-washed (4 N HCl, 1 h, 65°C), 18 MΩ deionized water-rinsed, Teflon®-coated forceps. The samples were transferred to certified Hg-free screw-capped vials, immediately frozen on dry ice for transport to Montana State University, and stored at −50°C in a laboratory freezer. One-half of the larval specimens collected from each spring were later thawed and dissected with acid-washed dissecting instruments in a laminar-flow hood in a clean room. The foregut, midgut and hindgut and their contents from three larvae from Hazle Lake Spring, four larvae from Succession Spring and eight larvae from Dragon Spring were transferred to certified Hg-free vials, producing a single sample of pooled gut material from multiple specimens for each spring. Tracheal tissue was collected from the same larvae from which gut was collected and pooled in a manner similar to gut in certified Hg-free vials. The vials and contents were stored at −50°C. The remaining larval specimens from each spring were split into two equal portions: one portion was used to determine dry weight/wet weight of intact larvae while the larvae in the remaining portion were dissected and their gut and tracheal tissue used for dry weight/wet weight determination.

Collection and processing of spring water for Hg analysis

A sample of spring water adjacent to the mat in Frying Pan Bowl and Frying Pan West Springs collected on 28 September 2005, a sample of spring water adjacent to mat grazed by the larvae in Succession and Dragon Springs collected on 18 April 2006, and a sample of interstitial water from the mat grazed by larvae in Hazle Lake Spring collected on 22 September 2006 were transferred to pre-cleaned (4 N HCl, 12 h, 65°C; 18 MΩ deionized water-rinse) Teflon® or glass bottles with Hg-free certification containing trace element-free hydrochloric acid as a preservative using a peristaltic pump and fresh acid-leached C-flex® pump-head tubing and Teflon® tubing with an in-line pre-combusted (4 h, 550°C) Whatman G/FC quartz fibre filter (0.4 μm nominal pore size) for each water sample to remove particulate matter as described previously (King *et al*., 2006). A sample of interstitial water from the mat grazed by larvae as well as a second sample of water adjacent to the mat in Succession Spring was collected on 17 May 2006 and transferred to pre-cleaned Teflon® or glass bottles containing trace metal-free hydrochloric acid preservative for $MeHg^+$ analysis or trace metal-free nitric acid/dichromate preservative for THg analysis using the pump system described above except that a fresh, in-line pre-cleaned, all-plastic filter unit (0.1 μm pore size) was substituted for the glass fibre filter to remove particulate material from each sample.

Mercury analysis

Samples were processed and analysed at the USGS Wisconsin Mercury Research Laboratory, Madison, WI or at Frontier Geosciences, North Seattle, WA. Aqueous phase $MeHg^+$ was analysed using chromatographic separation with cold vapour atomic fluorescence spectrometry according to previously cited protocols (DeWild *et al*., 2002). Methylmercury was extracted from larval gut and tissue samples and feather by additions of KBr, $CuSO_4$ and methylene chloride and analysed according to previously cited protocols (DeWild *et al*., 2002). THg in aqueous, larval gut and tissues samples was analysed according to previously cited protocols (Olson and DeWild, 1999) with the following modifications: samples were digested with HNO_3/H_2SO_4 prior to addition of BrCl.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Taxonomic affiliation of unclassified Yellowstone National Park stratiomyid larvae and selected members of the family *Stratiomyidae* calculated by the maximum-likelihood method. The bar represents 0.005% nucleotide substitution.

Table S1. Mercury in biotic and abiotic compartments of various aquatic systems containing elevated levels of mercury.

Appendix S1. Reference sequences used in analysis of the stratiomyid 28S rDNA sequence and method of construction of phylogenetic tree in Fig. S1.

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