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Obligate groundwater crustaceans mediate biofilm interactions in a subsurface food web

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Abstract: Food webs in groundwater ecosystems are dominated by only a few top-level consumers, mainly crustaceans. These obligate groundwater dwellers–or stygobites–clearly interact with groundwater biofilm, but it is uncertain whether they affect the abundance and structure of biofilm assemblages. We hypothesized that crustacean stygobites would reduce bacteria and protozoan abundance and alter biofilm assemblage structure. We also hypothesized that high densities of stygobites would remove more bacteria and protozoa than would low densities, and that this difference would become more pronounced over time. First, we established that the amphipods *Niphargus fontanus* and *Niphargus kochianus* both ingest biofilm by examining their gut contents. We then conducted two microcosm experiments. The first experiment showed that both *N. fontanus* and the isopod *Proasellus cavaticus* increased protozoan abundance but that bacterial abundance was only slightly reduced in the presence of *P. cavaticus*. In the second experiment, we determined how zero, low, and high densities of *N. kochianus* affected the biofilm. The high-density treatment of *N. kochianus* had significantly higher protozoan abundance than the control and the low-density treatment, and high densities of *N. kochianus* significantly increased the relative proportions of small and medium-sized bacteria over time compared with controls. Our controlled microcosm experiments demonstrate that macroinvertebrate stygobites can influence groundwater biofilm assemblages, although the exact mechanisms are not clear. These results support the hypothesis that stygobites influence essential ecosystem services supplied by groundwater ecosystems.

Keywords: protozoa, microcosms, bacteria, biofilm, flow cytometer, stygobite, *Niphargus*, *Proasellus*.

Groundwater is a critical resource for the ~2 billion people worldwide who depend on it for drinking water (Morris et al. 2003). Moreover, many terrestrial and aquatic ecosystems rely wholly or partially on access to groundwater (Boulton 2005). Biotic communities within groundwater contribute to the maintenance of groundwater quality via the breakdown of organic matter, nutrients, and contaminants (e.g. Kota et al. 1999, Gibert and Deharveng 2002, Tomlinson & Boulton 2008) providing vital ecosystem services (Griebler and Avramov 2015). Many of the resident animals (called stygobites) are groundwater obligates (Gibert et al. 1994), and they uniquely contribute to global biodiversity. Stygobite species often have restricted distributions (Gibert et al. 2009), which make them especially vulnerable to anthropogenic pressures such as pollution (Boulton et al. 2003).

Food webs in groundwater ecosystems are also unique in that they are truncated and far less complex than their surface water counterparts. Their simplicity is associated with the negligible primary production in most groundwater ecosystems, which are largely dependent on scarce allochthonous energy sources to fuel community biomass and production (Gibert et al. 1994, Gibert and Deharveng 2002). Organic matter is the basal component of these food webs; prokaryotes, single-celled eukaryotes (protozoans); and microscopic metazoans are primary consumers; and macroinvertebrates (principally crustaceans) or cavefish are top-level consumers. In comparison with their surface water counterparts, stygobites have a reduced metabolism and low growth and reproduction rates – adaptations to the limited energy and constant temperature in the groundwater environment (Spicer 1998). Other stygobite adaptations include lack of eyes and pigmentation and resistance to hypoxia and starvation (Hervant et al. 1995, Hervant et al. 1999).

Groundwater food web interactions, especially those between micro- and macroorganisms, are poorly understood (Griebler and Avramov 2015, but see Boulton et al. 2008). Few experimental studies with appropriate replication have been conducted.

Conflicting evidence exists for whether or not stygobitic crustaceans cause top-down control in groundwater food webs. Cooney and Simon (2009) found that *Gammarus minus*, a cave amphipod, reduces bacterial activity, whereas other studies demonstrated that bacteria are more abundant and active when grazed by *G. minus* or *Caecidotea tridentata*, a subterranean isopod (Edler and Dodds 1996, Kinsey et al. 2007). Other studies have found no consumptive effects of stygobites (Foulquier et al. 2010, 2011). Researchers mainly attributed this lack of effect to low metabolic rates and low abundances of top-level consumers in energy-limited environments (Foulquier et al. 2010, 2011). It seems likely that grazer density and feeding time are important predictors to consider when investigating the effects of stygobites on groundwater assemblages. Similarly, there is contradictory evidence for bottom-up control of groundwater food webs. Foulquier et al. (2010, 2011) found that bacterial assemblages were more abundant and active at higher levels of dissolved organic carbon (DOC). However, Weitowitz (2017) found that higher nutrient concentrations did not result in higher bacterial abundances.

Trophic relationships in surface water ecosystems have received considerable attention in recent decades (e.g. Sih et al. 1985, Billen and Servais 1990, Muylaert et al. 2002, Shurin et al. 2012). These studies clearly show that both bottom-up and top-down forces are important in structuring biological communities (McQueen et al. 1989, Menge 2000). Macrofaunal isopod and amphipod crustaceans such as *Gammarus* spp. and *Asellus* spp. are known to play a critical role in surface waters both as food for higher trophic levels and as decomposers of organic material (Graca et al. 1994a, 1994b). These taxa can also affect biofilm groups such as small metazoans (Rosemond et al. 2001) and algae (Duffy and Hay 2000, Bruno et al. 2008), but they are not known to purposefully predate on protozoans. However, surface water protozoans can strongly influence bacterial populations in both positive and negative ways (e.g. Wey et al. 2012, Huws et al. 2005, Humphreys 2009). Given

101 the importance and strength of consumer-mediated interactions in surface waters it is likely
102 that such interactions also occur in groundwater ecosystems.

103 In addition to feeding interactions, aquatic invertebrates can have indirect effects on
104 the microbial food web and ecosystem functioning. For example, macrofauna are known to
105 both bioturbate sediments and compact fine sediments into fecal pellets (Boulton et al.,
106 2008). Furthermore, interstitial bacterial activity can be stimulated by invertebrate
107 bioturbation in sediments (Mermillod-Blondin et al. 2000), and microbial activity can be
108 enhanced through nutrients provided by hyporheic invertebrates in the form of fecal pellets
109 (Boulton 2000, Marshall and Hall 2004).

110 Macrofaunal invertebrate stygobites are the top consumers in many groundwater
111 ecosystems. However, amphipods and isopods move and appear to acquire food differently.
112 The amphipods *N. fontanus* (Bate 1859) and *N. kochianus* (Schellenberg 1932) preferentially
113 use their gnathopods to pick up, manipulate and ingest pieces of sediment. The isopod *P.*
114 *cavaticus* (Leydig 1871), however, is a bottom crawler, directly grazing on sediment surfaces
115 (personal observation). Previous authors showed that sedimentary biofilm provides up to 83%
116 of the diet for *P. cavaticus* (e.g. Francois et al. 2016). However, the evidence is less clear for
117 *Niphargus* spp., which have been described as being both polyphagus (Fiser et al. 2008,
118 Arnscheidt et al. 2012) and predatory (Knight and Johns 2015).
119
120 In this study, we tested two hypotheses: (1) The presence of stygobites will
121 significantly reduce bacterial and protozoan abundances and alter biofilm assemblage
122 structure. *Proasellus cavaticus* will exert a stronger effect than *N. fontanus* because of its
123 scraping 'lawn mower' feeding strategy, which has also been observed in some surface
124 isopods (Naylor 1955, Jones 1972). (2) High stygobite densities will remove more bacteria

125 and protozoans than low densities, and this effect will become more pronounced over time as
126 fewer and fewer reproductive bacteria and protozoa remain in the system.
127
128 **METHODS**
129 To test our hypotheses, we first quantified the diets of the 3 target species. We then
130 conducted 2 manipulative experiments.
131 **Study species**
132 All 3 target species are 8 – 11 mm long and commonly occur in the UK. *Niphargus*
133 *kochianus* (Fig. 1A) is the most abundant and widespread amphipod species in UK chalk
134 aquifers (Maurice et al. 2016). The isopod, *P. cavaticus* (Fig. 1B), occurs mainly in carbonate
135 aquifers (Johns et al. 2015). *N. fontanus* (Fig. 1C) is found in a wide range of groundwater
136 habitats in the UK (Johns et al. 2015).
137
138 **Gut content study**
139 We conducted a preliminary study to confirm that the *Niphargus* species used in our study
140 feed on and ingest sedimentary biofilm. We collected 45 individuals of *N. kochianus* and 2 *N.*
141 *fontanus* from a chalk borehole (Berkshire, UK) and then starved the animals in ultrapure
142 water for 14 days to promote gut clearing. We then incubated individuals (one per
143 microcosm) with a biofilm-coated stone tile (Fiji, B&Q, dimension - 3.1 x 1.4 x 0.8 cm) at 11
144 °C in the dark for 96 h. These tiles were previously exposed to groundwater for 4 weeks to
145 allow the natural colonization of biofilm. Tiles were placed in the same chalk borehole used
146 to source the stygobite amphipods. Individuals were then stored in > 98 % ethanol. Those
147 that had expelled their guts on preservation were discarded. We followed the approach of
148 Navarro-Barranco et al. (2013) to better observe gut contents. Specimens were placed in
149 vials of Hertwig's liquid (270 g of chloral hydrate, 19 mL of 1N chloric acid, 60 mL of

glycerine, and 150 mL of distilled water) in an oven at 65 °C for 4 hours. Individuals were then mounted on a slide and the contents of the foregut (we were only interested in food intake over the last 96 hours) studied under an Olympus BX53 microscope and photographed at x400 magnification.

Experiment 1. Testing the hypothesis that stygobite presence will reduce bacterial and protozoan abundances and alter biofilm assemblage structure.

Experimental setup and design Nine *N. fontanus* and 9 *Proasellus cavaticus* were collected over 2 days in November 2013 from a cave system in Wales (Elm Hole; latitude 51.81, longitude -3.14) and kept in the dark in containers of cave water at 11 ℃.

We exposed stone tiles in a borehole (chalk, Berkshire, UK) to obtain natural groundwater biofilms. Stone tiles of equal size (Fiji, B&Q, dimension - 3.1 x 1.4 x 0.8 cm) were autoclaved and washed in ultrapure water, placed in mesh nets with a mesh diameter of 500 µm, and suspended in the borehole for 3 weeks to colonize. Griebler et al. (2002) showed that numbers of attached bacteria on sediment in similarly clean groundwater near Salzburg, Austria reached 500 * 10^5 cells per cm^3 within 4 weeks of exposure. On retrieval, tiles were transported to the laboratory in a cool box and stored in unfiltered groundwater in the dark at 11 ℃ (the same temperature as water in the borehole) for four weeks until the start of the experiment, which allowed for further growth of the biofilm.

For this experiment, we used 3 treatments (consumer *N. fontanus*, consumer *P. cavaticus*, and a control) each with 27 replicates (3*27=81 microcosms). We used a block design running 6 replicates on days 0 to 4 (Run 1), another 6 replicates on days 8 to 12 (Run 2), and another 6 on days 16 to 20 (Run 3). For the last block, we ran nine replicates on day 24 (Run 4) (see Table S1). We employed this design because we had to 're-use' individuals to obtain a high replication. This temporal block design enabled us to statistically account for

any differences in starting conditions such as the condition of the biofilm tiles (Bailey and Reiss 2014). One individual represented one replicate in each of the 4 runs (e.g. *N. fontanus* individuals 1 to 6 and *P. cavaticus* individuals 1 to 6 were used for day 0-4 (see Table S1). All individuals were used 3 different times—twice in the 4-day trials (runs 1-3) and once in run 4).

Prior to each experimental run, the crustaceans were starved in filtered groundwater for 4 days to allow them to empty most of their intestines. Only animals with empty foreguts were used in the experiments. Microcosms were set up in 50 mL glass beakers containing 20 mL of filtered and autoclaved borehole water and were kept at 11 °C in darkness to mirror groundwater conditions. One tile was placed in each microcosm to provide a food source for the stygobites, and 1 individual of each species was introduced into the respective treatments. Stygobites were checked for mortality every 24 h (two died during the experiment and were replaced with an individual of equal size on discovery).

Each run was terminated after 96 h. We then retrieved crustaceans from the microcosms, measured the abundance of bacteria and protozoa on the tiles, and assessed the structure of each biofilm community.

Response variables We used a toothbrush to brush the biofilm on each tile into 10 mL of 0.25 µm filtered, autoclaved water, a widely used method to detach biofilm from various substrates (see Wipfli et al. 1998, Cardinale et al. 2002, Bouletreau et al. 2006, Vercraene-Eairmal et al. 2010). We used 10 standardized downstrokes on each side of the tiles. We then homogenized the samples with a magnetic stirrer before further processing.

To assess the protozoa, we fixed two 500-µl subsamples of the homogenate for microscopic analysis in 2% glutaraldehyde. We used a gridded Sedgwick Rafter cell to count and measure protozoa in each sample under an Olympus CX 21 microscope at x400

magnification. We followed Adl et al. (2006) to assign all protozoan cells to 10 morphotype categories, including different types of ciliates, flagellates, and testate amoebae. We used Foissner and Berger (1996) to aid in protozoan identification and morphotype assignments.

For the bacterial analysis, we poured a 1 mL subsample of the initial homogenate through a 40-µm filter. We used a C6 flow cytometer (BD Technologies, North Carolina) to analyze 495-µl of this filtrate. Preliminary trials in which samples were both sonicated and homogenized resulted in significantly higher counts of non-bacterial debris but did not significantly increase bacterial counts (Weitowitz 2017). We therefore chose not to use sonification to further separate clumps of bacterial cells. Preliminary trials (Weitowitz 2017) also helped us determine the best possible threshold level to identify bacteria and exclude noise. The primary threshold was set at SSC-H (side scatter) 4000 and a secondary threshold at FSC-H (forward scatter) 8000. A dual threshold applies more stringent conditions before counting a particle and excludes more potential noise (BD Biosciences, 2011, p. 5).

We used SYTO-9 (Molecular Probes, Life Technologies; Massachusetts) to stain bacteria and distinguish them from soil particles (Lebaron et al. 1998, Gasol and Del Giorgio 2000). After preliminary staining trials (Weitowitz 2017), we selected a final SYTO-9 concentration of 5 µM (see also Lebaron et al. 1998). We mixed 495 µl of microcosm homogenate with 5 µl of SYTO-9 stock solution resulting in a total volume of 500 µl for flow cytometric analysis. After adding stain, we incubated the samples in the dark at room temperature for 15 minutes to allow the stain to bind to the DNA.

Before counting bacteria, we gated out noise caused by the applied electrical voltage and the running of filtered water using FSC-H vs FL-1 (green fluorescence) dot plots (Troussellier et al. 1999). We kept these bacterial gates constant throughout the experiment. Different bacterial size groups were identified according to their clustering along the FL-1 fluorescence axis, allowing for a discrimination of different bacterial populations (see

225 Troussellier et al. 1999). We then ran each 500 µl sample for 1 minute at slow flow to
226 minimize doublet counts.
227
228 **Experiment 2. Testing the hypothesis that high densities will remove more bacteria and**
229 **protozoans than low densities and that this effect will become more pronounced over**
230 **time.**
231 ***Experimental setup and design*** For the second experiment, we collected 250 individuals
232 of *N. kochianus* from two boreholes in the Berkshire Chalk aquifer. Collected animals were
233 transported to the laboratory in a cool box filled with groundwater that was maintained at 11
234 °C. In one of the boreholes we suspended 2 tile sizes (Fiji, B&Q, large = 3.1 x 1.4 x 0.8 cm,
235 small = 1.5 x 1.5 x 1 cm) in mesh bags to allow groundwater biofilm to colonize over a
236 period of 5 wk. Next the tiles were stored for 4 wk in the dark at 11 °C until the start of the
237 experiment. This storage period allowed additional growth of the biofilm.
238 This experiment featured 3 treatments: ungrazed biofilm as a control, 'low *Niphargus*
239 density' and 'high *Niphargus* density'. We used nine *N. kochianus* for the low-density
240 treatment and 18 individuals for the high-density treatment. The densities were based on
241 invertebrate sampling (standardized net hauls) conducted in the same chalk aquifer
242 (Weitowitz 2017). Each treatment had 10 replicates, resulting in 30 microcosms (Table S2).
243 To create the microcosms, we filled 250-mL glass beakers with 100 mL of filtered
244 and autoclaved groundwater. We placed 2 large rectangular tiles for bacterial analysis and six
245 small tiles for protozoan analysis in PARAFILM-sealed microcosms. A single control tile in
246 a mesh bag (mesh size 0.1 mm^2) was suspended in all microcosms of the treatments and
247 control, which the crustaceans were not able to access. This tile was used to assess biofilm
248 dynamics in the absence of grazing. We then added the stygobites. Over the course of 32
249 days, we sampled protozoans from 5 random replicates of each treatment on six occasions

(days 2, 5, 11, 16, 23, 32 for a total of 90 samples). Bacteria were sampled in all replicates on 9 occasions (days 2, 3, 5, 9, 11, 16, 18, 23, 27, 32 for a total of 270 samples).

Response variables We obtained samples for protozoan analysis by sacrificing one small tile on each sampling occasion. We carefully brushed the biofilm on each protozoan tile into 10 mL of autoclaved water by applying 10 standardized downstrokes with a toothbrush. We then fixed samples with glutaraldehyde and counted protozoa under a microscope as in experiment 1.

We sampled bacteria from two large tiles each marked by a grid of 15 evenly sized (0.6 x 0.4 cm) sections (Weitowitz 2017). On each sampling occasion, we pooled three 200-µl samples directly pipetted from randomly selected sections in each microcosm, and we ensured that no section was sampled more than once. After pipetting, clear patches became visible on the tiles suggesting that biofilm was present and was sampled effectively. The bacterial samples were then thoroughly homogenized in Eppendorff tubes, before being processed in the flow cytometer as in experiment 1. We assigned each counted bacterium to one of 3 body size categories: small, medium and large.

Statistical analyses

We performed all statistical analyses in the open source statistical environment R (R Development Core Team 2013). Initially all response variables were checked for normality and homogeneity of variance with the Shapiro-Wilk normality and Levene variance tests. If a response variable violated parametric assumptions, we used the Box-Cox transformation method of package 'MASS' (Venables and Ripley 2002) to identify the best form of power-transformation for the dependent variable.

For the first experiment, we assessed if differences in protozoan and bacterial abundance occurred between the 3 treatments (see Table 1). Because we reused individuals in this experiment and because the experiment was run in blocks (see Table S1), we analyzed the data with linear mixed effects models (LMMs) in the R package 'lme4' (Bates et al., 2015). LMMs are commonly used to analyze ecological data when multiple measurements (e.g. on a single individual) constitute pseudoreplicates (Perkins et al. 2012, Zuur et al.2009). Because we 'reused' individual stygobites three times, we fitted individual ID as a random effect to account for differences in which particular individuals affect the biofilm. We also fitted Block as a predictor in the models, because some of the replicates were run on different days. The R-code for the LMM was: lmer(log10(Response) ~ Consumer + Block + (1| Individual). We used a Tukey post-hoc test to find out which treatments were different from each other.

In the second experiment we also used LMMs in the R package 'lme4' (because this approach is identical to repeated measures ANOVA) to test for the effect of different stygobite densities, time, and their interaction on bacterial and protozoan response variables. The R-code was: lmer(Response ~ Treatment*Day + (1 | Unique ID), where Treatment is high, low, or zero (control) *Niphargus* density, and Unique ID is the microcosm that was measured repeatedly over time, represented by the variable Day. We used a Tukey test for post-hoc comparisons.

RESULTS

Gut content analyses

The gut content analyses established that both *Niphargus* species ingested biofilm. We found a homogeneous mass of recently ingested organic material (e.g. bacteria, protozoans) and sediment particles in the foreguts of the *Niphargus* individuals (Fig 1C-F).

299 All individuals initially had empty foreguts, indicating that the material found came from
300 biofilm associated with the tiles. Overall, we detected organic material in 32 of the 45 *N.*
301 *kochianus* individuals and both of the *N. fontanus* individuals.
302
303 **Experiment 1. Hypothesis: Stygobite presence will reduce bacterial and protozoan**
304 **abundances and alter biofilm assemblage structure.**
305 The presence of both *N. fontanus* and *Proasellus cavaticus* had a significant positive
306 effect on protozoan abundances found on tiles (Table 1, Fig. 2) compared with the control
307 without stygobites. In *N. fontanus* and *P. cavaticus* microcosms, the number of protozoans
308 was double that of the control (Fig. 2). The post-hoc test for protozoan abundance showed
309 that the effects of both species were different from the control (Tukey-test; *Niphargus* vs
310 control, $P<0.01$ and *Proasellus* vs control, $P<0.05$). In the LMM, the random effect explained
311 only 1% of protozoan abundance, i.e. the identity of the individual stygobite used was not a
312 significant predictor of the response.
313 The effect of stygobites on bacterial abundances was less marked (Table 1). Although
314 *P. cavaticus* seemed to reduce the number of bacteria (Fig. 3), this effect was not significant
315 and variation in bacteria abundance was much greater between blocks (Table 1). Neither
316 stygobite species changed the bacterial assemblage structure in terms of altering the relative
317 proportion of small, medium, and large bacteria (data not shown). Block had a highly
318 significant effect on bacterial abundance (Table 1). For example, bacterial abundance was
319 significantly lower in block 4 than in block 1, indicating that bacterial abundance changed
320 significantly with time. Thus, it was important to fit Block as a predictor in the LMMs.
321
322 **Experiment 2. Hypothesis: High densities will remove more bacteria and protozoans**
323 **than low densities and this effect will become more pronounced over time.**

As in the first experiment, protozoan abundances were significantly affected by the density of *N. kochianus,* by time, and by the interaction between density and time (Table 2). As with the other two stygobites, the presence of *N. kochianus* at high densities resulted in more protozoans than in the control treatment (Fig. 4). In fact, when averaged across all time points and density treatments, protozoan abundances were twice as high when *Niphargus* was present (Fig. 4). However, these differences did not occur during the first part of the experiment. Abundances remained at comparably low levels in all treatments from day 2 to day 16 (Fig. 4). However, from day 23 on, protozoan abundance increased in the high density *N. kochianus treatment* relative to the control (Fig. 4).

No significant differences in the number of protozoan morphotypes occurred across treatments, but the number of protozoan morphotypes in all treatments increased significantly over time (Table 2, Fig. 4).

The density treatments did not significantly affect bacterial abundance (Table 2). However, bacterial assemblage structure was significantly affected by *N. kochianus* density, by time, and by the interaction of the two predictors. '*Niphargus* Density' was a significant predictor of the proportion of small and medium bacteria, but not of large bacteria (Fig. 5, Table 2). On day two of the experiment, small bacteria tended to make up a larger proportion of the total bacterial population in the high-density treatment relative to either the low-density or control treatment (Fig. 5). Conversely, the initial relative proportions of medium and large bacteria tended to be higher in the low-density and control treatments (Fig. 5). Throughout the course of the experiment the relative proportions of small, medium, and large bacteria continuously changed. The percentage of small bacteria decreased in the high-density treatment, while the proportion of medium and large bacteria tended to increase (Fig. 5). In the low-density and control treatments, the proportion of medium and large bacterial size

348 classes tended to slightly decline over time. By day 32, the proportion of bacterial size classes
349 was very similar between treatments (Fig. 5).

350 On the mesh tiles excluded from stygobite access, bacterial abundance ($F_{2,243} = 0.5$, P
351 > 0.05) and the proportion of small ($F_{2,243} = 0.1$, P > 0.05), medium ($F_{2,243} = 0.02$, P > 0.05)
352 and large bacteria ($F_{2,243} = 0.06$, P > 0.05) did not differ between treatments.
353
354 **DISCUSSION**
355 Our experiments showed that the *Niphargus* species can ingest biofilm and that the
356 presence of each of the three species altered the biofilm. The strength and nature of this effect
357 depended on stygobite density and the duration of exposure to the biofilm.
358 Our microcosm experiments offer a unique glimpse of macroinvertebrate stygobite
359 behavior and their influence on primary resources within experimental microcosms.
360 However, our experimental design did not enable us to determine whether these are direct
361 food web effects, facilitation via increased nutrient recycling, or a combination of processes.
362 The role of stygobites in groundwater food webs has been intensely debated in recent years
363 (e.g. Boulton et al. 2008). Despite their widespread prevalence and the absence of other top-
364 level consumers, most studies have attributed little importance to obligate groundwater
365 animals, because of the temporal stability of groundwater ecosystems and the low metabolic
366 rates and perceived low abundance of stygobites (Gibert et al. 1994, Boulton et al. 2003,
367 Wilhelm et al. 2006, Sorensen et al. 2013). However, controlled experiments investigating
368 groundwater food webs are scarce (but see Edler and Dodds 1996, Cooney and Simon 2009,
369 Foulquier et al. 2010).
370
371 **Effects on Protozoa**

372 Both single individuals of *N. fontanus* and *P. cavaticus*, as well as *N. kochianus* at
373 high densities, significantly increased protozoan abundance in our experimental microcosms.
374 As there is currently little information on the role of stygobites in groundwater food webs, the
375 consistency of this effect across all experimental species is noteworthy. It remains to be
376 determined whether the stimulatory link to protozoans is mediated directly by feeding activity
377 or indirectly via excretion or bioturbation.

378 Previous studies have shown that microscopically small surface-water crustaceans
379 such as copepods and cladocerans selectively feed on specific protozoan species (Sanders and
380 Wickham 1993, Reiss and Schmid-Araya 2010) and size classes (Stoecker and Capuzzo
381 1990, Sommer et al. 2001), thus demonstrating that these crustaceans can actively target
382 protozoans. Stygobites are also thought to obtain their nutrients from biofilm coating
383 sediments and rocks, including associated protozoans (Baerlocher and Murdoch 1989,
384 Fenwick et al. 2004, Boulton et al. 2008). However, for our study species, predation on
385 protozoans does not appear to be substantial, given that predators tend to reduce prey
386 abundances (Sih et al. 1985, Mamilov et al. 2000) and protozoan abundance did not decline.
387 It is possible that rapid turnover and recruitment of Protozoa completely compensated for
388 losses due to predation. Another possibility is that stygobites may either bioturbate or graze
389 the biofilm, causing tightly bound biofilm fragments to be dislodged from the substratum
390 (e.g. Gibert et al. 1994). These activities would provide a greater surface area for grazing by
391 bacterivorous protozoans, allowing them to reproduce faster and attain higher abundances.

392 Stygobite presence increased morphotype diversity in experiment 2. Protozoans such
393 as flagellates and ciliates are omnipresent in groundwater (Novarino et al. 1997), so the
394 resting spores (Finlay 2002) of many protozoan species would have been present on the
395 biofilm tiles. However, it seems that when stygobites were absent, the spores remained

396 dormant. It is possible that the proliferation of protozoans (caused by stygobites) increased
397 the likelihood that rarer protozoan morphotypes would be detected in our subsamples.
398
399 **Effects on bacteria**
400 Only *P. cavaticus* reduced bacterial abundances in experiment 1, and the effect was
401 not strong compared with changes detected for protozoans. The gut contents of both
402 *Niphargus* species show they clearly ingest tile-associated biofilm. This result indicates that
403 bacteria in biofilm are likely to provide at least some of the diet for stygobites (Boulton et al.
404 2008). However, previous studies have found both strong positive and negative correlations
405 between bacterial responses and stygobite grazing (Griebler et al. 2002, Cook et al. 2007,
406 Foulquier et al. 2010, 2011).
407 In experiment 2 we measured respiration rates from 5 replicates of one small tile in all
408 treatments (reported in Weitowitz 2017). We measured respiration both halfway through and
409 at the end of the experiment. The bacterial activity rates were higher in the presence of
410 stygobites, perhaps because either their grazing or bioturbation removed senescent bacteria
411 and enhanced solute uptake by active bacteria. Such effects may explain the relatively small
412 difference in bacterial abundances between stygobite treatments (Weitowitz 2017). The
413 relatively small amount of bacterial biomass removed by invertebrate and protozoan grazing
414 might be offset by the increase in bacterial growth. Other studies in surface waters and
415 terrestrial ecosystems have also shown an effect of higher-order animals on bacterial activity
416 rates across a range of taxa, including collembolans (Hanlon and Anderson 1979), nematodes
417 (Traunspurger et al. 1997), and protozoans (Hahn and Hoefle 2001).

418 We also observed time-dependent effects on bacterial assemblage structure. These
419 effects might be a direct result of stygobite grazing, an indirect effect associated with
420 increased protozoan grazing, or both given that protozoans were more abundant in the

421 presence of stygobites. In other aquatic systems, protozoan grazing is size-selective
422 (Chrzanowski et al. 1990, Gonzalez et al. 1990, Simek and Chrzanowski 1992) and has been
423 shown to affect bacterial assemblage structure (Hahn and Hoefle 1999, 2001). For example,
424 the uptake efficiency of bacteria by flagellates and ciliates, the dominant protozoans in our
425 biofilm, decreases with prey cell size. No lower uptake limit exists (Hahn and Hoefle 2001).
426 Stygobites consume microbes (Simon et al. 2003, Hallam et al. 2008), so they may also
427 directly affect bacterial assemblage structure. In the presence of stygobites, small and
428 medium-sized bacteria were initially present at lower frequencies than large-sized bacteria,
429 but this pattern quickly disappeared. One explanation for this observation is that the smaller
430 sizes of bacteria responded by increasing their activity and rate of cell division. Such
431 compensatory reactions in response to predation have been observed previously and were
432 attributed to rapid bacterial generation rates (Hanlon and Anderson 1979, Traunspurger et al.
433 1997).

434 Both *N. fontanus* and *P. cavaticus* increased protozoan abundance in the biofilm, but
435 only *P. cavaticus* reduced bacterial abundance and only slightly. These responses may have
436 been at least partly caused by the different feeding strategies of the species, with *P. cavaticus*
437 harvesting the bacterial 'carpet' more efficiently than *N. fontanus*. However, both species
438 appear to increase the nutrient availability to protozoans, but through different behaviors.
439 *Proasellus cavaticus* may dislodge biofilm by browsing over sediment and scraping off
440 bacteria, whereas *N. fontanus*, an active swimmer, may dislodge biofilm via bioturbation as it
441 passes over and disturbs the sediments.

442 The relationships between components in groundwater food webs are not limited to
443 organismal interactions. Stygobites also provide food directly to microbes and protozoa by
444 excreting feces or producing pellets of fine interstitial materials (Boulton et al. 2008). We
445 observed these activities in our experimental microcosms. Bacteria are known to process

446 fecal pellets in aquatic habitats (e.g. Yoon et al. 1996, Wotton and Malmqvist 2001), and this
447 activity may partly explain how the bacteria overcame increased grazing pressure. In the
448 control microcosms, however, the nutrient-poor conditions in combination with the reduced
449 nutrient cycling likely provided unfavorable conditions for bacterial reproduction.

450 Aquifers and their associated organisms, particularly protozoa and bacteria, support
451 important ecosystem services such as nutrient (e.g. denitrification, nitrification) and
452 contaminant transformation (e.g. biodegradation) (Mattison et al. 2002, 2005, Tomlinson and
453 Boulton 2008). They also maintain carbon flux through food webs. The effect of stygobites
454 on groundwater biofilm demonstrated here could have important implications for these
455 services, and stygobytes may also play a significant role in maintaining clean drinking water.
456 Future studies should address these important issues.

457

458 **CONCLUSIONS**

459 Our experiments suggest that stygobites can increase abundances of protozoa and
460 alter the structure of both protozoa and bacteria assemblages. As for species from surface
461 ecosystems, their impact is likely to depend on their abundance in the systems. To date,
462 however, estimates of stygobite abundance in aquifers are rare (Maurice and Bloomfield
463 2012, Sorensen et al. 2013).

464 Maintaining groundwater ecosystem functionality and stability is becoming
465 increasingly important in the face of environmental pollution and global climate change.
466 Groundwater biota, and particularly stygobites, are adapted for the constant temperature and
467 low-nutrient conditions in groundwater. A change in groundwater temperatures or nutrient
468 levels could therefore lead to the disappearance of whole functional groups of organisms in
469 these simple systems, leading to ecosystem destabilization (Avramov et al. 2013). Further
470 experiments are needed to identify the mechanisms by which stygobites affect groundwater

biofilms and influence ecosystem services, and thus build a foundation for an informed approach to the conservation of these systems.

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737 **FIGURE CAPTIONS**

738 Fig. 1. Photos of the stygobite grazer species (A) *Niphargus fontanus*, (B) *Proasellus*
739 *cavaticus* and (C) *N. kochianus.* Panels C–F show the gut content of *N. kochianus.*
740 These contents are shown at x400 magnification in (E) and (F) and reveal a
741 homogeneous mass of organic material and sediment particles.

742 Fig. 2. The effect of control, *N. fontanus*, and *P. cavaticus* treatments (one individual per
743 replicate) on protozoan abundances (individuals / mL H_2O) in feeding microcosms
744 (experiment 1). The box and whisker plots summarize replicates from four different
745 experimental time blocks, with individual data points superimposed to visualize the
746 distribution of the data. The horizontal line within the box indicates the median and
747 the boundaries of the box indicate the 25^{th} and 75^{th} percentiles.

748 Fig. 3. The effect of control, *N. fontanus*, and *P. cavaticus* treatments (one individual per
749 replicate) on bacterial abundances (individuals / µL H_2O) in feeding microcosms
750 (experiment 1). The box and whicker plots summarize replicates from four different
751 experimental time blocks, with individual data points superimposed to visualize the
752 distribution of the data. The horizontal line within the box indicates the median and
753 the boundaries of the box indicate the 25^{th} and 75^{th} percentiles.

754 Fig. 4. The effect of control and different density treatments (low and high) of *N. kochianus*
755 on protozoan abundance (individuals / mL^{-1} H_2O) and number of protozoan
756 morphotypes (number / mL) over time in experiment 2. Different density treatments
757 are symbolized by dotted (control), dashed (low density), and solid (high density)
758 lines. Protozoan responses were sampled on six occasions (days 2, 5, 11, 16, 23 and
759 32).

760 Fig. 5. The effect of control and different density treatments (low and high) of *N. kochianus*
761 on the relative proportion of small, medium, and large bacterial size classes (as % of

762 total bacteria) over time in experiment 2. Different density treatments are symbolized
763 by dotted (control), dashed (low density), and solid (high density) lines. Bacteria
764 responses were sampled on nine occasions (days 2, 3, 5, 9, 11, 16, 18, 23, 27, 32).