International Journal of Speleology	42 (2)	141-145	Tampa, FL (USA)	May 2013

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International Journal of Speleology

Official Journal of Union Internationale de Spéléologie

Exploring the sulfide tolerance of ectosymbiotic *Niphargus* amphipods from the Frasassi caves, central Italy

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Abstract: Two species of the crustacean amphipod genus *Niphargus* inhabit the sulfidic groundwaters of the Frasassi caves in central Italy, and both harbor filamentous, sulfide-oxidizing *Thiothrix* ectosymbionts. As sulfide is toxic to most aerobic organisms, it appeared possible that the ectosymbionts could help their *Niphargus* hosts with detoxification processes. In this study, mortality due to sulfide was compared between *Niphargus* individuals with ectosymbionts and individuals whose ectosymbionts had been killed by antibiotic treatment. Both Frasassi-dwelling *Niphargus* species revealed exceptionally high tolerances to sulfide compared to other amphipod species studied so far. *Niphargus* individuals without viable ectosymbionts tolerated sulfide levels exceeding those occurring in Frasassi cave waters. Thus, the amphipods may employ *Thiothrix*-independent mechanisms for sulfide resistance.

Keywords: ectosymbionts; Frasassi caves; *Niphargus*; sulfide tolerance; *Thiothrix*

Received 8 April 2013; Revised 7 May 2013; Accepted 9 May 2013

Citation: Bauermeister J., Assig K. and Dattagupta S., 2013. Exploring the sulfide tolerance of ectosymbiotic *Niphargus* amphipods from the Frasassi caves, central Italy. International Journal of Speleology, 42 (2), 141-145. Tampa, FL (USA) ISSN 0392-6672
http://dx.doi.org/10.5038/1827-806X.42.2.6

INTRODUCTION

The Frasassi caves are located in the Apennine Mountains of the Marche region in central Italy and are actively forming by sulfuric acid-driven limestone dissolution (Galdenzi, 1990). Sulfide in the cave waters fuels primary productivity by chemoautotrophic microbes, which supports the food web of the ecosystem (Sarbu et al., 2000). Sulfide is toxic to most invertebrates, as it binds to cytochrome *c* oxidase and inhibits mitochondrial electron transport (Nicholls, 1975). Nevertheless, crustacean amphipods of the species *Niphargus ictus* and *Niphargus frasassianus* live in the sulfidic Frasassi cave waters (Flot et al., 2010). Both species harbor filamentous, sulfide-oxidizing *Thiothrix* ectosymbionts (Dattagupta et al., 2009; Bauermeister et al., 2012).

A variety of invertebrates living in sulfide-rich marine habitats employ effective strategies, partially involving microbial symbionts, to avoid sulfide poisoning (Cavanaugh et al., 2006; Dubilier et al., 2008). Deep-sea tubeworms and shallow-water clams host intracellular endosymbionts that can oxidize hydrogen sulfide to non-toxic sulfur compounds (Anderson et al., 1987; Wilmot & Vetter, 1990). A sulfide-detoxifying role has further been proposed for ectosymbiotic bacteria covering the cuticles of stilbonematid nematodes (Hentschel et al., 1999) and for those on the gill surfaces of hydrothermal-vent shrimp (Tokuda et al., 2008). On the other hand, it has been argued that the diffusion rate of sulfide into the bodies of oligochaete worms and other invertebrates far exceeds the rate at which it can be oxidized by their ectosymbionts (Dubilier et al., 1995; Ruehland & Dubilier, 2010). Thus, whether or not sulfur-oxidizing ectosymbionts can assist their hosts in preventing sulfide poisoning remains debated.

In the case of Frasassi-dwelling *Niphargus, Thiothrix* ectosymbionts are located on the walking legs of the animals, which are close to the amphipod gills (Bousfield, 1978). It is thus conceivable that the ectosymbionts might diminish sulfide diffusion into the amphipods' bodies (Dattagupta et al., 2009). The aim of the present study was to determine whether ectosymbionts of *N. ictus* and *N. frasassianus* increase the sulfide tolerances of their hosts. For this purpose, we treated individuals of both *Niphargus* species with antibiotics to kill their ectosymbionts and subsequently exposed *Thiothrix*-hosting and *Thiothrix*-lacking *Niphargus* to cave water whose sulfide content was gradually increased until lethal concentrations were reached.

MATERIALS & METHODS

In July 2010, 24 *N. ictus* and 24 *N. frasassianus* individuals were collected from the Frasassi cave lake Lago Verde and from the turbulent cave stream

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Sorgente del Tunnel, respectively (for a map of the Frasassi caves, please refer to Bauermeister et al., 2012). Animals were caught using small fishing nets and forceps, as appropriate, and transferred into polypropylene bottles filled with cave water from the respective sampling site. Additionally, non-sulfidic water was collected from the Frasassi cave pool Il Bugianardo (BG). *Niphargus* individuals and cave water samples were kept at ambient cave temperature during transfer to the laboratory.

The experiment was conducted at the Osservatorio Geologico di Coldigioco field station in Frontale di Apiro, in a basement room which typically has ambient temperatures of 13-22 °C. Three of the 24 *N. ictus* individuals died during transfer, so experiments were conducted with only one instead of four *N. ictus* control individuals (details below). An antibiotic solution (12.5 mg/L) was prepared from streptomycin sulfate (Roth, Karlsruhe, Germany) dissolved in filter-sterilized BG cave water. Eleven of the 21 *N. ictus* and 14 of the 24 *N. frasassianus* individuals were incubated in the solution for 24 h in order to kill their *Thiothrix* ectosymbionts. Streptomycin was chosen as the antibiotic agent due to its high effectiveness against *Thiothrix* as demonstrated in a previous study (Williams & Unz, 1985). After the antibiotic treatment, *Niphargus* individuals were immersed in filter-sterilized BG cave water to wash off any excess streptomycin.

Five beakers, hereafter referred to as B1-B5, were filled with a layer of autoclaved limestone gravel covered by 500 mL BG cave water (sterilized by filtration through 0.2-micron membranes). Antibiotically treated and non-treated *Niphargus* were divided into B1-B5 as shown in Fig. 1. Antibiotically treated *N. ictus* (N=1) and *N. frasassianus* (N=4) individuals in B5 served as control animals to check whether the streptomycin treatment or other experimental parameters would cause mortality in the absence of sulfide.

A sulfide stock solution (SSS) was prepared from 60 mg of sodium sulfide nonahydrate ($Na_2S \cdot 9H_2O$; SIGMA-ALDRICH, Steinheim, Germany) dissolved in 45 mL of filter-sterilized BG cave water. Starting one hour after placing the animals in the beakers, a sterile pipette was used to add several milliliters of SSS to B1-B4 at approximately hourly intervals for 13 hours (12 additions in total). The pipette tip was dipped into the water and slowly stirred while releasing the SSS to ensure uniform mixing and prevent rapid oxidation of the sulfide by oxygen (Chen & Morris, 1972). The same volume of filter-sterilized, non-sulfidic BG cave water was added in a similar manner to B5, serving as control treatment. The number of living *Niphargus* in the beakers was determined before each addition of SSS. Dissolved sulfide concentrations in the waters were measured with a DR 2800 spectrophotometer using the methylene blue method (HACH LANGE, Düsseldorf, Germany) approximately 15 min after each addition of SSS. Simultaneously, dissolved oxygen, pH, and temperature in the waters were determined using HQ40d multimeter sensors (HACH LANGE). After the third addition of SSS, increasing pH-values of the incubation waters were repeatedly adjusted to ~8 by addition of a few drops of concentrated hydrochloric acid (Table 1).

After completion of the experiment, six antibiotically treated and five non-treated *Niphargus* individuals were prepared for examination using scanning electron microscopy (SEM). The specimens were transferred into individual vials filled with 2.5% glutaraldehyde solution (SIGMA-ALDRICH) made in filter-sterilized BG cave water. They were later sequentially dehydrated in ethanol concentrations from 30% to 90%, with a final dehydration in hexamethyldisilazane (SIGMA-ALDRICH) for 5-10 min. The samples were mounted on carbon-coated aluminum sample holders, sputtered with gold-palladium (11 nm thickness), and examined with a LEO 1530 GEMINI field emission SEM (Zeiss, Göttingen, Germany).

Both *N. ictus* and *N. frasassianus* are reported to be endemic to the Frasassi cave ecosystem (Karaman, 1986; Karaman et al., 2010). Due to concerns about threatening their potentially delicate populations, we used a limited number of individuals of each species for our experiment. This precluded replication of the various treatments and statistical analyses of our data.

RESULTS & DISCUSSION

SEM revealed that non-treated *Niphargus* individuals harbored numerous intact *Thiothrix* filaments, whereas animals treated with streptomycin prior to the experiment featured empty filament sheaths or remnants of *Thiothrix* holdfasts still attached to their exoskeletons (Fig. 2). These observations confirmed the effective killing of *Thiothrix* ectosymbionts by the antibiotic treatment. Temperatures in the incubation waters ranged between 20 and 22 °C, which is 7-9 °C above Frasassi cave water temperatures (Macalady et al., 2006). However, all control animals in B5

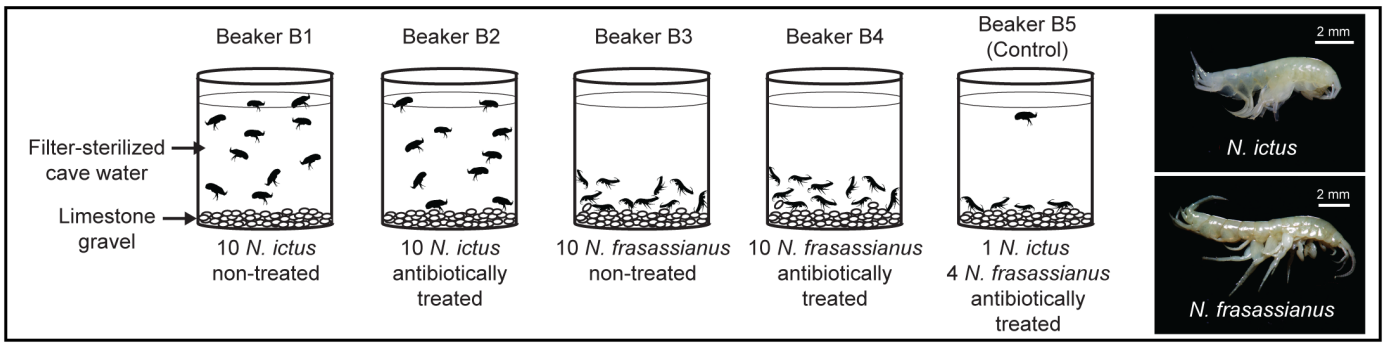

Fig. 1. Experimental set-up of this study.

Table 1. Overview of the results of the sulfide exposure experiment. Full-length vertical lines indicate time points of sulfide addition.

	Time [h:min]	0:00	1:00	2:15	4:00	5:30	6:45	7:45	8:45	9:45	10:45	12:30	13:15	14:00
Beaker B1 *N. ictus* non-treated	Sulfide [mM]	0.0	0.3	1.1	0.6	0.5	1.0	2.1	3.4	2.5	3.9	7.4	7.4	14.3
	Oxygen [µM]	236	231	201	134	100	80	63	13	85	14	7	32	22
	Number of animals alive	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**3**
Beaker B2 *N. ictus* antibiotically treated	Sulfide [mM]	0.0	0.1	0.3	0.5	0.5	1.1	1.7	4.2	3.6	5.9	9.2	12.0	16.3
	Oxygen [µM]	236	227	214	158	133	108	82	29	45	17	7	14	6
	Number of animals alive	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**9**	**8**	**8**	**8**	**7**	**0**
Beaker B3 *N. frasassianus* non-treated	Sulfide [mM]	0.0	0.3	0.5	0.5	0.6	1.0	2.0	3.9	4.4	6.6	11.1	9.8	13.0
	Oxygen [µM]	236	224	195	143	117	94	58	14	16	20	47	52	13
	Number of animals alive	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**0**
Beaker B4 *N. frasassianus* antibiotically treated	Sulfide [mM]	0.0	0.2	0.4	0.5	0.5	0.9	1.5	3.2	3.7	5.5	7.4	11.5	11.4
	Oxygen [µM]	236	234	195	135	112	82	64	32	33	19	10	12	9
	Number of animals alive	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**6**	**2**
Beaker B5 control animals antibiotically treated	Sulfide [mM]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Oxygen [µM]	236	254	254	235	252	249	247	246	247	242	239	238	237
	Number of animals alive (*N. ictus* + *N. frasassianus*)	**1+4**	**1+4**	**1+4**	**1+4**	**1+4**	**1+4**	**1+4**	**1+4**	**1+4**	**1+4**	**1+4**	**1+4**	**1+4**

survived the entire experiment (Table 1), implying that temperature did not play a defining role in mortality.

All antibiotically treated *N. ictus* and *N. frasassianus* individuals survived sulfide concentrations of 1.7 mM and 1.5 mM, respectively (Table 1). These concentrations are more than three times as high as those measured in Frasassi cave waters (0.1 to 0.5 mM sulfide; Galdenzi et al., 2008). Thus, the *Thiothrix* ectosymbionts of *N. ictus* and *N. frasassianus* are probably not essential for preventing sulfide poisoning of their hosts in the cave waters. At sulfide levels between 3 and 7 mM, we observed that two antibiotically treated *N. ictus* died, whereas all untreated individuals survived (Table 1). Although we cannot rule out that the ectosymbionts provided a survival advantage at these high sulfide levels, it is perhaps more likely that the antibiotic treatment weakened the *Niphargus*, making them less resistant to high sulfide and/or low oxygen concentrations.

Amphipods generally have low sulfide tolerances (Theede et al., 1969; Oseid & Smith, 1974; Sandberg-

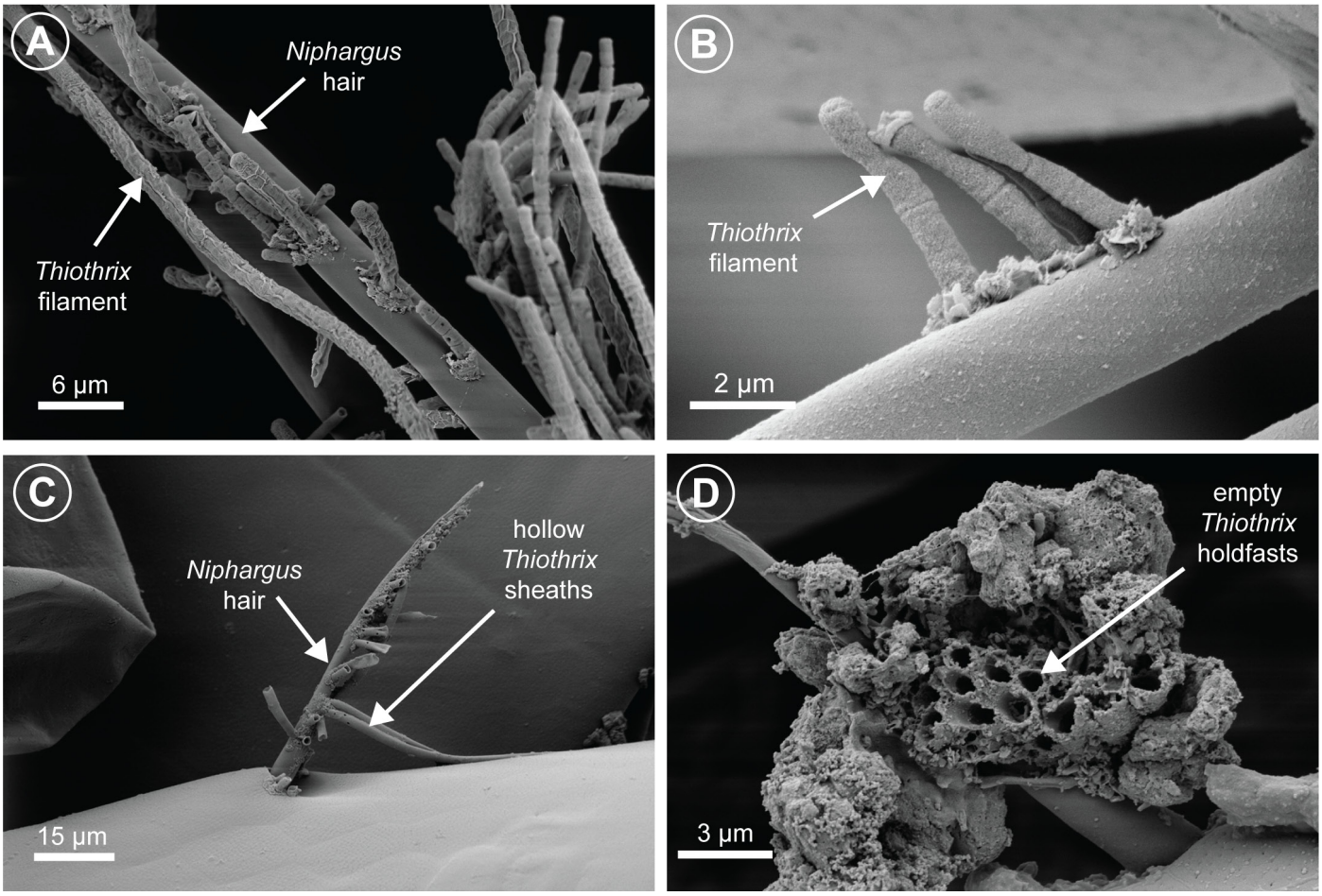

Fig. 2. Scanning electron micrographs of intact filaments and remnants of *Thiothrix* ectosymbionts on *Niphargus*. Panels A and B: Intact ectosymbiotic *Thiothrix* filaments on *Niphargus* (images are of *Niphargus* individuals not subjected to the sulfide experiment). Panels C and D: Empty *Thiothrix* sheaths and holdfasts on *Niphargus* individuals treated with streptomycin solution.

Kilpi et al., 1999). Knezovich et al. (1996) found that all 20 individuals of the infaunal amphipod *Rhepoxynius abronius* used for their experiment had died after 48 h exposure to 78 µM sulfide, and 14 out of 20 individuals of the infaunal amphipod *Eohaustorius estuarius* had died after 48 h exposure to 116 µM sulfide. In our experiment, all 20 non-treated *N. ictus* and *N. frasassianus* individuals survived sulfide concentrations as high as 7 mM (Table 1). To the best of our knowledge, this is to date the highest experimentally determined sulfide concentration tolerated by a crustacean (Vaquer-Sunyer & Duarte, 2010). Frasassi-dwelling *Niphargus* could presumably employ symbiont-independent sulfide detoxification processes. Crustaceans are commonly not able to exclude sulfide from their bodies, but they can oxidize it to non-toxic thiosulfate or sulfite. Detoxification is either mediated enzymatically by sulfide oxidase (Vetter et al., 1987) or proceeds via oxygen-independent sulfide binding to metallic ions (Vismann, 1991). Additionally, mitochondrial sulfide oxidation has been suggested to occur in the muscle tissue of certain crustaceans (Vismann, 1991; Johns et al., 1997).

CONCLUSION

N. ictus and *N. frasassianus* amphipods living in sulfide-rich waters of the Frasassi cave system appear to have exceptionally high sulfide tolerances compared to other crustaceans studied to date. However, their sulfide-oxidizing *Thiothrix* ectosymbionts are unlikely to play a critical role in sulfide detoxification. It remains to be examined whether these bacteria provide the *Niphargus* with other benefits.

ACKNOWLEDGEMENTS

The authors thank Alessandro Montanari for providing lab facilities and logistical support at the Osservatorio Geologico di Coldigioco. Sincere thanks are also given to Anne Wilkening for assistance during the sulfide experiment, and to Dorothea Hause-Reitner and Wolfgang Dröse for assistance with scanning electron microscopy. The study was funded by the German Initiative of Excellence. This is publication number 119 from the Courant Research Center Geobiology of the University of Göttingen.

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