

**Diversity and ecology of ectosymbioses
between sulfur-oxidizing *Thiothrix* bacteria
and groundwater niphargid amphipods**

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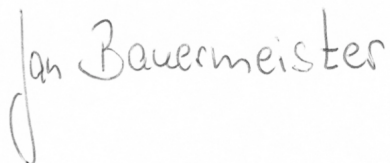
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Versicherung

Hiermit versichere ich an Eides statt, dass die Dissertation mit dem Titel
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Introduction

Thiothrix

Thiothrix Winogradsky 1888 is a genus of filamentous, sulfur-oxidizing bacteria belonging to the family Thiotrichaceae. The generic name derives from the Neo-Greek words "theion" ("sulfur") and "thrix" ("hair") (Euzéby, 1997). *Thiothrix* filaments (trichomes) are composed of rod-shaped cells separated by transverse septa. The cells can vary largely in size and shape (0.7–4.0 μm in diameter and 0.7–5.5 μm in length; Aruga *et al.*, 2002) and may be encased by a polysaccharide sheath (Takeda *et al.*, 2012; Figure I.1).

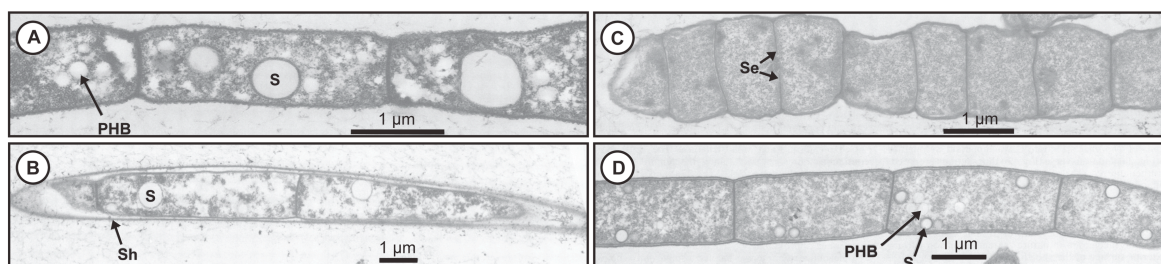


Figure I.1. Thin sections of trichomes of the *Thiothrix* species *T. unzii* (A), *T. fructosivorans* (B), and *T. eikelboomii* (C and D). The images illustrate the high morphological variety of *Thiothrix* cells, even of those belonging to the same species (C and D). Abbreviations: PHB = polyhydroxybutyrate, S = sulfur, Sh = sheath, Se = septum. (Reproduced with permission from Williams *et al.* (1987), *Applied and Environmental Microbiology* 53: 1560–1570, ©American Society for Microbiology).

Thiothrix bacteria are found in marine as well as freshwater environments (Unz and Head, 2005). While individual *Thiothrix* filaments are colorless, free-living populations form white biofilms. They are usually attached to solid surfaces in sulfide-containing flowing waters with around neutral pH-values. Numerous strains have been isolated from activated-sludge wastewater treatment reactors, where excessive *Thiothrix* growth causes sludge bulking (Jenkins *et al.*, 2003).

The life cycle of *Thiothrix* has been proposed by Larkin and Shinabarger (1983). *Thiothrix* filaments are non-motile, but they can release rod-shaped cells called gonidia from their tips, when environmental conditions become unfavorable for their growth. The gonidia can disperse in water by gliding movements and attach to a new substratum via monopolar fimbriae (Williams *et al.*, 1987). After attachment, the

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gonidia produce a holdfast from which a new trichome grows out. Most *Thiothrix* strains form rosette-like filament structures that arise from the accumulation of multiple gonidia at the attachment site.

Thiothrix are commonly described as aerobic or microaerophilic bacteria (Unz and Head, 2005), but it has been suggested that some strains may be capable of using nitrate instead of oxygen as an electron acceptor under anoxic conditions (Nielsen *et al.*, 2000). Cultivated *Thiothrix* strains have been shown to grow on a wide range of organic and inorganic carbon substrates (Larkin and Shinabarger, 1983; Odintsova *et al.*, 1993; Tandoi *et al.*, 1994; Howart *et al.*, 1999; Aruga *et al.*, 2002; Rossetti *et al.*, 2003; Chernousova *et al.*, 2009). They need a reduced inorganic sulfur compound as an energy source and electron donor for chemolithoautotrophic growth (Table I.1). Heterotrophic strains also use the additional energy obtained from sulfur oxidation to increase their growth rates through facultative mixotrophy.

Table I.1. Designation of organisms according to their metabolic capabilities (after Madigan *et al.*, 2003). *Thiothrix* bacteria are metabolically very versatile, and the capability of chemoautotrophic, chemoheterotrophic, and mixotrophic growth has been demonstrated for various strains. Mixotrophy (not listed) is the ability to switch between or use a combination of different energy, electron, or carbon sources (Eiler, 2006).

Energy source	Electron donor	Carbon source	Name
Light	Inorganic compounds	Carbon dioxide	Photolithoautotroph
		Organic compounds	Photolithoheterotroph
	Organic compounds	Carbon dioxide	Photoorganoautotroph
		Organic compounds	Photoorganoheterotroph
Chemical reaction	Inorganic compounds	Carbon dioxide	Chemolithoautotroph
		Organic compounds	Chemolithoheterotroph
	Organic compounds	Carbon dioxide	Chemoorganoautotroph
		Organic compounds	Chemoorganoheterotroph

When provided with a reduced sulfur compound, for example hydrogen sulfide (H₂S), *Thiothrix* bacteria oxidize that compound to elemental sulfur, which is deposited as granules within invaginations of their cell membranes (Figure I.1). In times of sulfur deficiency in the environment, the sulfur can be oxidized to sulfate (SO₄²⁻; Larkin and Strohl, 1983), which provides *Thiothrix* with cellular energy. It has also been suggested that the sulfur storages may serve as terminal electron acceptors,

when oxygen and nitrate are not available (Nielsen *et al.*, 2000). When grown on acetate, several heterotrophic and mixotrophic *Thiothrix* strains further store polyhydroxybutyrate granules as carbon resources in their cells (Larkin and Shinabarger, 1983; Howarth *et al.*, 1999; Rossetti *et al.*, 2003; Figure I.1).

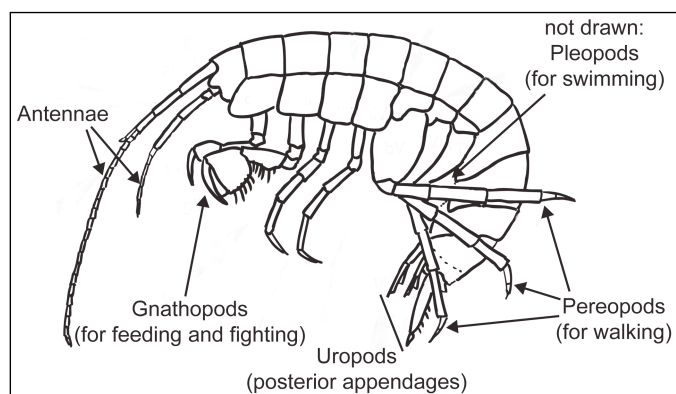
Gammaridean amphipods

Gammaridea is a suborder of small, shrimp-like crustaceans in the order Amphipoda. It contains over 90% of all described amphipod species, divided among around 125 families (Foster *et al.*, 2009).

Gammaridean amphipods have an elongate body, which is segmented throughout and more or less laterally compressed. They undergo direct development without a larval stage. Females carry their embryos in a brood pouch (marsupium) located ventrally between their thoracic legs. When the juveniles get released, they reach maturity after several moltings (= sheddings of their exoskeletons).

Most gammarideans are aquatic. They have their widest distribution in the marine environment, but can also be found in a large spectrum of brackish and freshwater habitats. Freshwater species predominantly live epi- or nektobenthic, whereas marine taxa can also be pelagic, commensalistic, or parasitic (Väinölä *et al.*, 2008). Feeding habits of gammarideans are diverse; there are scavengers, predators, suspension- and detritus-feeders, commensals, parasites, and farmers (Stoddart *et al.*, 2003).

Figure I.2. Body structure of a gammaridean *Niphargus* amphipod. The name "Amphipoda" means "legs on both sides" (Jaeger, 1955) and refers to the distinctly shaped appendages of the animals. (Modified and reprinted from Encyclopedia of Caves, 2nd Edition, Cene Fišer, *Niphargus: a model system for evolution and ecology, part I: key properties*, pp 556–559, Copyright (2012), with permission from Elsevier).



With over 300 known species, *Niphargus* Schiødte 1849 (Figure I.2) is the largest genus of freshwater gammaridean amphipods (Fišer *et al.*, 2008). Most *Niphargus*

species inhabit subterranean environments and constitute a major component of the groundwater biodiversity in Europe (Sket, 1999). Characteristic of animals adapted to life in permanent darkness, *Niphargus* lack eyes and pigmentation (Ginet, 1960; Gibert, 1977). Congruously, the name "*Niphargus*" refers to the Greek word *niphargês* meaning "white like snow" (Flot *et al.*, 2010a).

Symbioses

In 1879, the German mycologist Heinrich Anton de Bary introduced the term symbiosis for "the living together of differently named organisms" (De Bary, 1879). Today, many researchers describe a symbiosis as lifetime or at least long-lasting interspecies relationship that eventuates in benefits for at least one of the involved organisms. Symbioses affect all levels of biological organization (Paracer and Ahmadjian, 2000; Moran, 2006); they have played a key role in the origin of eukaryotic cells (Sagan, 1967; Gray *et al.*, 1999; Bhattacharya *et al.*, 2007) and are vital for virtually every metazoan life form on Earth. Thus, the evolutionary and ecological significance of symbioses cannot be overestimated.

Symbioses include facultative relationships between equally autonomous organisms as well as obligate associations of closely connected, interdependent species. The benefits derived from symbioses are manifold, including protection (Fautin and Allen, 1997; Heil and McKey, 2003) and improved nutrient supply (Breznak and Brune, 1994; Dilworth *et al.*, 2008). Yet other symbioses are indispensable for reproduction processes (Dedeine *et al.*, 2001) or the completion of life cycles (Herre *et al.*, 1996).

A reciprocally beneficial symbiosis is called mutualism. Commensalism describes a relationship from which one of the involved partners benefits, whereas the other remains unaffected. If an organism reaps profits out of a symbiosis and thereby harms its partner, parasitism occurs. The character of a symbiosis is not fixed but may change according to evolutionary adaptations of the organisms engaged, so that mutualism may evolve towards parasitism and vice versa (Herre *et al.*, 1999; Sachs and Simms, 2006). Likewise, the same organism might act as a beneficial partner in one symbiosis, but as a parasite in another (Aanen and Hoekstra, 2007).

Chemosynthetic animal-bacteria symbioses

Chemosynthetic microorganisms derive energy from the oxidation of reduced chemicals and convert carbon from carbon dioxide or methane into biomass (Dubilier *et al.*, 2008). They can thrive in complete darkness and sustain whole ecosystems that receive no input of photosynthetically produced organic matter from the Earth's surface (Sarbu *et al.*, 1996; Sievert and Vetriani, 2012). Numerous invertebrates living in reducing marine environments harness chemosynthesis via symbioses with sulfur-, methane-, and hydrogen-oxidizing bacteria (Cavanaugh *et al.*, 2006; Dubilier *et al.*, 2008; Petersen *et al.*, 2011).

Most sulfur-oxidizing bacteria rely on the availability of free oxygen, to which the electrons provided by sulfide or other reduced sulfur compounds are shuttled during oxidative phosphorylation (Cavanaugh *et al.*, 2006). However, sulfide and oxygen are mutually exclusive, as the former gets spontaneously oxidized in the presence of the latter (Almgren and Hagström, 1974). The two compounds co-exist only in very narrow zones close to the interface between oxygenated and anoxic water realms. Free-living sulfur-oxidizing bacteria thus have to assemble around these redox interfaces (chemoclines) in order to obtain both electron donor and acceptor (Jørgensen and Revsbech, 1983).

Motile invertebrates harboring sulfur-oxidizing symbionts reveal conspicuous behaviors by which they meet the metabolic needs of their bacterial partners. Thousands of shrimp of the species *Rimicaris exoculata* can be found coating the external walls of chimney-like black smokers at hydrothermal vents (Schmidt *et al.*, 2008). In those positions, the shrimp provide sulfur-oxidizing bacteria located on their mouthparts and within their enlarged gill chambers access to sulfide from the hot vent streams and to oxygen from the surrounding seawater. In reward, the ectosymbionts transfer organic compounds produced by chemoautotrophy transtegumentally to their host (Ponsard *et al.*, 2012). Shallow-water stilbonematid nematodes of the genera *Laxus*, *Eubostrichus*, and *Robbea* are covered by a coat of sulfur-oxidizing bacteria (Polz *et al.*, 1994, 1999; Bayer *et al.*, 2009). The worms migrate vertically between oxic and anoxic sediment layers and thereby expose their symbionts alternately to oxygen and sulfide. The bacteria, in turn, represent a ready food source for the worms (Ott *et al.*, 1991).

Sessile hydrothermal-vent invertebrates have sophisticated physiological adaptations to fuel inter- or intracellular sulfur-oxidizing symbionts. The giant vestimentiferan tubeworm *Riftia pachyptila* lacks both a mouth and a gut and is nutritionally dependent on chemoautotrophic bacteria housed in a specialized organ called trophosome (Stewart and Cavanaugh, 2006). The worm uses its gill-like branchial plume to take up oxygen and sulfide from the vent environment. Specialized hemoglobins in the plume's blood vessels can bind both chemicals separately and transport them to the bacteria in the highly vascularized trophosome. The tubeworm receives nutrients released by the bacteria or directly digests some of its endosymbionts. The clam *Calyptogena magnifica* uses its foot to dig for sulfide in vent cracks on the ocean floor and its siphon to gather oxygen from the oxygenated seawater (Childress *et al.*, 1991). While oxygen is bound to hemoglobin, sulfide links to a different protein in the blood serum, and both chemicals are transferred to nutrient-supplying symbionts in the clam's gill tissue (Zal *et al.*, 2000).

Chemosynthetic symbioses have been established independently in at least seven marine invertebrate phyla (Dubilier *et al.*, 2008). They are ecologically dominant only at deep-sea hydrothermal vents, but also occur at cold seeps, around whale and wood falls, and in organic-rich coastal sediments. Their wide distribution in the marine environment strongly contrasts their relative unrenownedness from freshwater habitats. Only a single example has been reported from sulfide-rich groundwaters of a terrestrial cave ecosystem (Dattagupta *et al.*, 2009).

The Frasassi caves

The Frasassi caves are located in the Apennine Mountains of the Marche region in central Italy. Near the small town of Genga (province of Ancona), the Sentino river cuts into the core of the Mount Frasassi-Mount Valmontagnana anticline and has shaped the 2 km long and 500 m deep Frasassi gorge. On both sides of this gorge, natural entrances to a network of more than 100 karst caves can be found (Sarbu *et al.*, 2000).

With over 20 km of interconnected passages, Grotta Grande del Vento-Grotta del Fiume is the largest Frasassi cave complex (Figure I.3). It is located on the south side

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of the Frasassi gorge and spans altitudes between 200 and 360 m above sea level. Dating of speleothems in the upper cave levels has revealed formation ages of up to 200,000 years (Taddeucci *et al.*, 1994). In the lower levels, cave development is still in progress and occurs roughly at the elevation of the Sentino river.

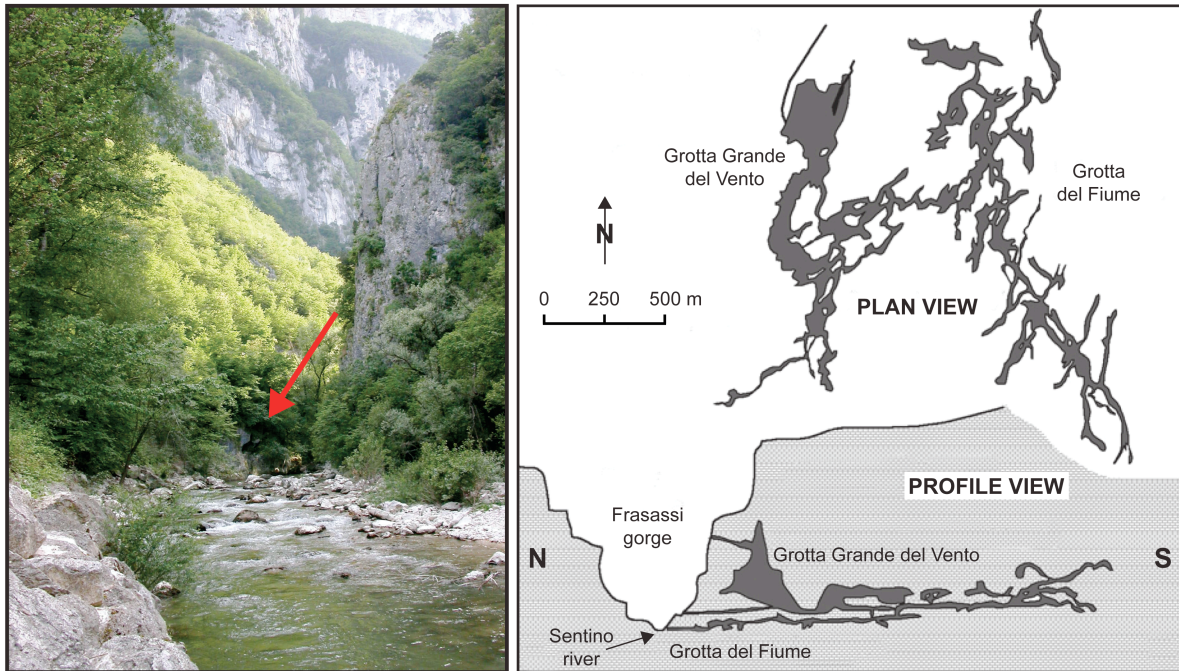
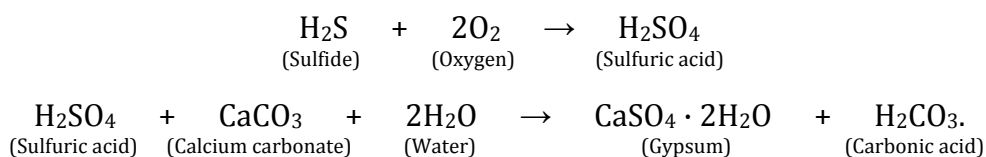


Figure I.3. Overview of the Frasassi Grotta Grande del Vento-Grotta del Fiume cave complex. Left: The Sentino river in the Frasassi gorge (direction of view: E-W). The red arrow points at the entrance to Grotta del Fiume. Right: Shadow maps of the cave complex. (Reproduced with permission from Hose and Macalady (2006), *New Mexico Geological Society Guidebook, 57th Field Conference, Caves and Karst of Southeastern New Mexico*, pp 185–194. ©New Mexico Geological Society).

The ongoing formation of Grotta Grande del Vento-Grotta del Fiume is due to sulfuric acid-driven limestone dissolution (Galdenzi, 1990). Sulfidic groundwater from a deep aquifer rises along a network of faults up to a thick limestone platform, where it mixes with oxygenated seepage water. Sulfide and oxygen react to form sulfuric acid, which further reacts with calcium carbonate in the host rock to form gypsum:



Below the cave water table, gypsum gets constantly dissolved, which results in the continuous growth of caverns in the rock. Hydrogen sulfide also degasses from the water table and reacts with atmospheric oxygen. Gypsum resulting from limestone dissolution in the vadose zone forms massive replacement crusts on the cave walls and ceilings (Galdenzi and Maruoka, 2003). Once the gypsum crusts have reached a critical thickness, they come off the walls, fall into the undersaturated cave water and get dissolved.

Chemosynthetic microorganisms substantially contribute to cave enlargement processes in Grotta Grande del Vento-Grotta del Fiume. Highly acidic, drop-shaped biofilms dangle from the gypsum-encrusted cave walls above sulfidic cave streams (Vlasceanu *et al.*, 2000). These so-called snottites belong to the lowest-diversity natural microbial communities known (Macalady *et al.*, 2007; Jones *et al.*, 2012). They are mainly composed of chemoautotrophic *Acidithiobacillus* bacteria that produce sulfuric acid as a byproduct of their sulfur-oxidizing metabolism.

Microbial mats of various morphologies are also present in many cave water locations (Macalady *et al.*, 2006). Cottony biofilms dominated by filamentous, sulfur-oxidizing *Beggiatoa* cover sediment surfaces in diffusion-controlled water niches. The gliding *Beggiatoa* can arrange themselves at the chemocline to get access to sulfide and oxygen. Non-gliding filamentous, sulfur-oxidizing bacteria are found in turbulent cave streams, where they form feathery biofilms attached to coarse sand particles and limestone boulders. The predominance of certain bacterial groups in these biofilms is determined by geochemical parameters. While epsilonproteobacteria dominate in flowing water niches with high sulfide-to-oxygen ratios, *Thiothrix* bacteria prevail at low sulfide-to-oxygen ratios (Macalady *et al.*, 2008).

Cave-wall and cave-water microbial communities in Grotta Grande del Vento-Grotta del Fiume constitute a plentiful food source for a rich metazoan cave fauna. 57 taxa of four different animal phyla, including molluscs, annelids, arthropods, and chordates, were identified in the course of an in-depth faunistic investigation in 1994 (Bertolani *et al.*, 1994). Roughly half of all species were troglaphiles, which are animals that can live both inside and outside caves. The majority of identified troglaphites – obligate cave-dwellers – were endemic to Frasassi. These included

gammaridean amphipods of the species *Niphargus ictus* Karaman 1985, representing the dominant macroinvertebrates in Frasassi cave waters (Figure I.4).

The *Niphargus-Thiothrix* symbiosis

Dattagupta *et al.* (2009) revealed that *Niphargus* individuals throughout Grotta Grande del Vento-Grotta del Fiume live in symbiosis with filamentous *Thiothrix* bacteria. Although the amphipods come in direct contact with a highly diverse microbial community in most cave waters, filaments of only a single *Thiothrix* phylotype were found attached to their chitinous exoskeletons (Figure I.4). Moreover, the ectosymbiotic phylotype was absent from or at least remarkably underrepresented in the microbial mats. The specific *Niphargus-Thiothrix* association was the first reported case of a chemosynthetic symbiosis from a non-marine habitat.

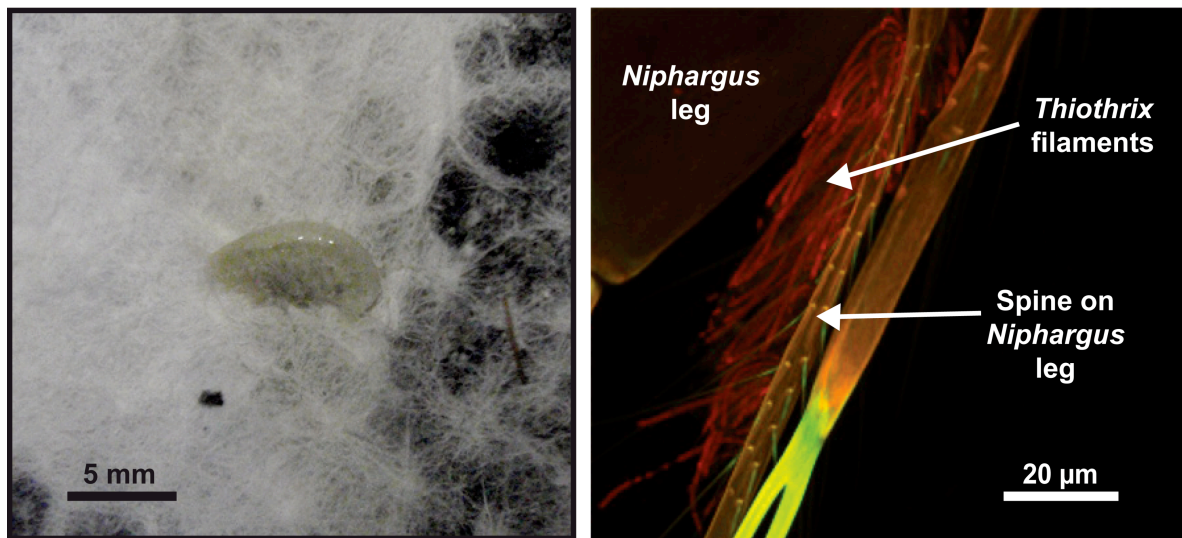


Figure I.4. The *Niphargus-Thiothrix* ectosymbiosis. Left: *Niphargus* amphipod in direct contact with mats of the highly diverse microbial community in Frasassi cave waters. Right: Confocal epifluorescence micrograph showing filaments of only a single *Thiothrix* phylotype (fluorescently marked by a phylotype-specific oligonucleotide probe; red) attached to a spine on a *Niphargus* leg. (Reprinted by permission from Macmillan Publishers Ltd: *The ISME Journal* (Dattagupta *et al.*, 2009), copyright (2009)).

In the study by Dattagupta *et al.* (2009), it was assumed that the *Niphargus-Thiothrix* symbiosis involves only one host species, as *N. ictus* was long-time said to be the only Frasassi-dwelling *Niphargus* species (Bertolani *et al.*, 1994; Sarbu *et al.*,

2000). Subsequent molecular and morphological investigations of *Niphargus*, however, revealed the presence of at least three different species in the cave system (Flot *et al.*, 2010a; Karaman *et al.*, 2010; Figure I.5). This finding called for a reexamination of the diversity, ecology, and distribution of the *Niphargus-Thiothrix* symbiosis and initiated the studies presented in this thesis.



Figure I.5. The three described *Niphargus* species from Frasassi. (A) *N. ictus* Karaman 1985, (B) *N. frasassianus* Karaman, Borowsky, Dattagupta 2010, and (C) *N. montanarius* Karaman, Borowsky, Dattagupta 2010. Photographs: Jean-François Flot.

Overview of the thesis chapters

This doctoral thesis contains four chapters, of which each describes a distinct study on a topic related to the above described *Niphargus-Thiothrix* ectosymbiosis. The chapters were written in the form of manuscripts, and the release status as well as a list of all contributing authors of each manuscript are given on the title page of the respective chapter. For ease of overview, reference lists of the manuscripts were included in the overall bibliography at the end of the thesis.

Regarding chapters 1–3, I contributed to the design of the studies, collected the samples, designed primers and probes, performed the experiments, analyzed the data, and wrote the manuscripts (with reviews by my supervisor). For the study described in chapter 4, I conducted scanning electron microscopy, designed *Thiothrix*-specific primers, constructed clone libraries, performed *Thiothrix*-specific PCR screenings and phylogenetic analysis of *Thiothrix* sequences, and wrote the manuscript parts referring to these analyses.

Chapter 1 describes the examination of the three Frasassi-dwelling *Niphargus* species for the presence and diversity of *Thiothrix* ectosymbionts. Using a combination of microscopic and molecular analyses techniques, we identified three

distinct, ectosymbiotic *Thiothrix* clades distributed among the *Niphargus* in a strongly host species-specific manner.

In **Chapter 2**, we investigated the metabolic characteristics of the three *Thiothrix* ectosymbionts on their different hosts and compared them to those of closely related free-living *Thiothrix* from Frasassi microbial mats. Conducting incubation experiments with isotopically labeled carbon substrates and nitrogen gas followed by Nano-scale Secondary Ion Mass Spectrometry (NanoSIMS), we found a remarkable metabolic diversity among the bacteria, and intriguing evidence for ecological benefits that *Thiothrix* derive from 'hitchhiking' on *Niphargus*.

Sulfide is toxic for most aerobic organisms. Nevertheless, two of the three Frasassi-dwelling *Niphargus* species live in sulfidic cave water. In **Chapter 3**, we considered the question whether the sulfur-oxidizing *Thiothrix* ectosymbionts protect their *Niphargus* hosts from sulfide poisoning. We exposed *Thiothrix*-bearing and *Thiothrix*-free *N. ictus* and *N. frasassianus* individuals to cave water with gradually increasing sulfide concentrations to find that the amphipods do not rely on their ectosymbionts for sulfide detoxification, but have an exceptionally high innate sulfide tolerance.

In **Chapter 4**, we expanded the search for *Thiothrix* ectosymbionts and examined *Niphargus* and *Pontoniphargus* amphipods from the sulfidic Movile cave and surrounding areas in the Dobrogea region of Romania. SEM and molecular analyses revealed the presence of *Thiothrix* belonging to two distinct phylogenetic clades on several Romanian niphargid species, suggesting that ectosymbioses between sulfur-oxidizing bacteria and groundwater amphipods may occur in sulfidic subterranean ecosystems throughout Europe.

Chapter 1:

Repeatedly evolved host-specific ectosymbioses between sulfur-oxidizing bacteria and amphipods living in a cave ecosystem

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1.1 Abstract

Ectosymbioses between invertebrates and sulfur-oxidizing bacteria are widespread in sulfidic marine environments and have evolved independently in several invertebrate phyla. The first example from a freshwater habitat, involving *Niphargus ictus* amphipods and filamentous *Thiothrix* ectosymbionts, was recently reported from the sulfide-rich Frasassi caves in Italy. Subsequently, two new *Niphargus* species, *N. frasassianus* and *N. montanarius*, were discovered within Frasassi and found to co-occur with *N. ictus*. Using a variety of microscopic and molecular techniques, we found that all three Frasassi-dwelling *Niphargus* species harbor *Thiothrix* ectosymbionts, which belong to three distinct phylogenetic clades (named T1, T2, and T3). T1 and T3 *Thiothrix* dominate the *N. frasassianus* ectosymbiont community, whereas T2 and T3 are prevalent on *N. ictus* and *N. montanarius*. Relative distribution patterns of the three ectosymbionts are host species-specific and consistent over different sampling locations and collection years. Free-living counterparts of T1–T3 are rare or absent in Frasassi cave microbial mats, suggesting that ectosymbiont transmission among *Niphargus* occurs primarily through inter- or intraspecific inoculations. Phylogenetic analyses indicate that the *Niphargus-Thiothrix* association has evolved independently at least two times. While ectosymbioses with T1 and T2 may have been established within Frasassi, T3 ectosymbionts seem to have been introduced to the cave system by *Niphargus*.

1.2 Introduction

Symbioses are vital for virtually all living organisms. They were critical for the origin and diversification of Eukaryotes and remain a major driving force in evolution, as they induce diverse physiological, morphological, and developmental modifications in the species involved (Sapp, 2004). Symbioses between invertebrates and chemosynthetic (e.g. sulfur- or methane-oxidizing) bacteria are of particular ecological importance in the marine environment, where they have evolved independently in at least seven metazoan phyla (Dubilier *et al.*, 2008). Many invertebrates living in sulfide-rich marine habitats, such as close to deep-sea hydrothermal vents, cold seeps, and in organic-rich coastal sediments, harbor sulfur-

oxidizing bacteria on their body surfaces (Dubilier *et al.*, 2008; Goffredi, 2010). Although the animals are exposed to diverse free-living microbial communities and therefore susceptible to colonization by many opportunistic, non-specific surface-dwellers (Wahl and Mark, 1999), many of them have established long-term and specific relationships with only few selected sulfur-oxidizing bacteria (Polz *et al.*, 1994; Goffredi *et al.*, 2004; Bayer *et al.*, 2009; Petersen *et al.*, 2010; Ruehland and Dubilier, 2010; Bulgheresi *et al.*, 2011). Most of these ectosymbionts belong to distinct groups within the epsilon- and gammaproteobacterial subdivisions. In particular, bacteria within the families Thiovulgaceae and Thiotrichaceae seem to have evolved an enhanced ability to establish ectosymbioses (Goffredi, 2010).

Thiothrix bacteria (family Thiotrichaceae) have been found as ectosymbionts on the marine amphipod *Urothoe poseidonis* (Gillan and Dubilier, 2004) and on the freshwater amphipod *Niphargus ictus* (Dattagupta *et al.*, 2009). The latter lives in sulfide-rich waters of the Frasassi caves (central Italy), which have been formed by sulfuric acid-driven limestone dissolution and contain an underground ecosystem sustained by chemoautotrophy (Sarbu *et al.*, 2000). Thick mats of filamentous sulfur-oxidizing epsilon- and gammaproteobacteria cover many of the cave water bodies (Macalady *et al.*, 2006, 2008). *Thiothrix* are abundant in these microbial mats, but the ectosymbionts of *N. ictus* are distinct from most of the *Thiothrix* bacteria found in the free-living communities (Dattagupta *et al.*, 2009).

At the time this symbiosis was discovered, *N. ictus* was reported to be the only amphipod species inhabiting the Frasassi cave ecosystem (Bertolani *et al.*, 1994; Sarbu *et al.*, 2000). However, subsequent molecular and morphological investigations revealed the presence of two additional species (Flot *et al.*, 2010a), which were named *Niphargus frasassianus* and *Niphargus montanarius* (Karaman *et al.*, 2010). Phylogenetic analyses suggested that the three *Niphargus* species most likely invaded the cave system independently (Flot *et al.*, 2010a). *N. frasassianus* and *N. montanarius* have so far never been observed to co-occur, but each of them has been found in sympatry with *N. ictus* at some locations within the Frasassi caves.

Host-related factors are considered to play a decisive role in ectosymbiont selection and maintenance (Chaston and Goodrich-Blair, 2010; Bright and Bulgheresi, 2010). It has recently been shown that stilbonematid nematodes of two different

genera living together in the same coastal marine sediments harbor distinct bacterial ectosymbiont phylotypes (Bulgheresi *et al.*, 2011). The *Niphargus* assemblage in Frasassi provided an opportunity to examine ectosymbiont specificity within partially sympatric, heterospecific members of the same invertebrate genus. In this study, all three Frasassi-dwelling *Niphargus* species were examined for *Thiothrix* ectosymbionts using a combination of Scanning Electron Microscopy (SEM), 16S rDNA sequencing, Fluorescence *In Situ* Hybridization (FISH), Automated Ribosomal Intergenic Spacer Analysis (ARISA), and nested-PCR. FISH was further used to inspect Frasassi microbial mats for free-living counterparts of the symbionts, and nested-PCR assays served for detecting symbiont dispersal cells. We report on three distinct *Thiothrix* ectosymbionts that are partially shared but yet distributed in a host species-specific manner among the *Niphargus*.

1.3 Materials and Methods

Sample collection & *Niphargus* species identification

Niphargus specimens were collected in January and May–June 2008, May–June 2009, July and October 2010, and March 2011 from within the Frasassi Grotta Grande del Vento-Grotta del Fiume complex at eight different cave locations (Il Bugianardo (BG), Grotta Sulfurea (GS), Sorgente del Tunnel (ST), Grotta Bella (GB), Lago Verde (LV), Pozzo dei Cristalli (PC), Ramo Sulfureo (RS), and Lago Claudia (LC); Figure 1.1). All sites were accessed via technical spelunking routes.

Niphargus species were determined in the field based on morphological characters described in Flot *et al.* (2010a) and Karaman *et al.* (2010). Individuals were caught using small fishing nets and forceps as appropriate. Specimens for SEM were collected into falcon tubes filled with cave water. They were later transferred to individual eppendorf tubes filled with a 2.5% glutaraldehyde solution made either in phosphate buffered saline (PBS) or in filter-sterilized cave water, and stored at 4 °C until analysis. Samples for clone library construction, FISH, ARISA, and nested-PCR assays were collected into individual eppendorf tubes filled with RNAlater® (Ambion/Applied Biosystems, Foster City, CA, USA) and stored at –20 °C until further analysis.

Microbial mat samples were obtained from Frasassi cave locations GS, ST, GB, PC, and RS in May–June 2009, and from locations Cave Spring (CS), GB, and RS in October 2011. They were collected into falcon tubes using sterile pipettes, preserved in 4:1 parts of RNAlater® within 4 h of collection, and stored at –20 °C until analysis.

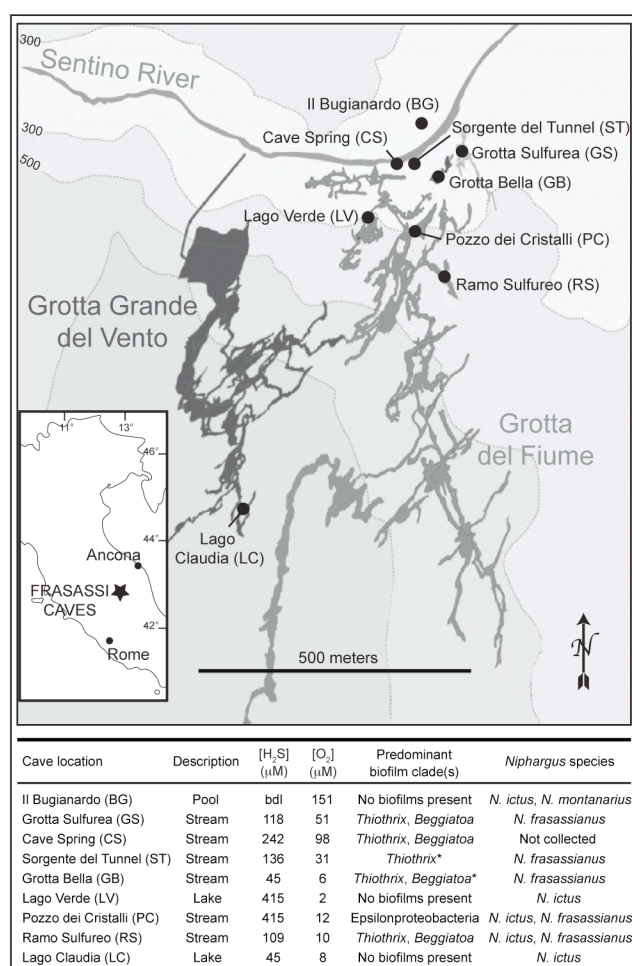


Figure 1.1. Map of the Grotta Grande del Vento-Grotta del Fiume complex of the Frasassi caves. Black circles in main map mark sample collection sites. Geochemistry data have been reported earlier by Flot *et al.* (2010a). Predominant biofilm clade determinations are based on FISH results (Macalady *et al.*, 2008), except for those marked with *, which were identified based on morphology (Macalady *et al.*, 2006). bdl = below detection limit. Base map courtesy of the Gruppo Speleologico CAI di Fabriano.

Scanning Electron Microscopy (SEM)

Two *N. frasassianus* individuals (locations GB and PC, June 2009), nine *N. ictus* individuals (location BG, June 2009 (1x), October 2010 (5x); location LC, May 2009 (1x); location LV, July 2010 (2x)), and one *N. montanarius* individual (location BG, June 2009) were investigated for *Thiothrix* epibionts using SEM. Whole specimens were sequentially dehydrated in ethanol concentrations from 30% to 90%, with a final dehydration in hexamethyldisilazane (SIGMA-ALDRICH, Munich, Germany) for 5–10 minutes. They 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).

DNA extraction

Niphargus appendages (legs and antennae) were dissected under a stereomicroscope. DNA extracts of *Niphargus* specimens collected in 2008 had previously been obtained from only two legs per individual (one gnathopod and one pereopod; cf. Flot *et al.*, 2010a). In order to increase the chance of gathering DNA from *Thiothrix* bacteria associated with *Niphargus*, DNA extractions for specimens collected from 2009 to 2011 were conducted with all appendages on one side of the *Niphargus* body. All extractions were performed using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions (starting with an overnight treatment with Proteinase K, followed by DNA precipitation and purification). Microbial mat DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions.

16S rDNA sequencing

16S rDNA clone libraries were obtained from five *N. frasassianus* samples (location GB, June 2008; location PC, May 2008; location RS, June 2008, May 2009; location ST, May 2009), two *N. ictus* samples (location BG, January 2008; location LC, May 2009), two *N. montanarius* samples (location BG, January 2008, June 2008), and one Frasassi microbial mat sample (location ST, May 2009). DNA was PCR-amplified using the bacterial domain-specific forward primer 27F and the universal reverse primer 1492R (both Weisburg *et al.*, 1991; see Supplementary Table S1.1 for sequences of all primers used in this study). The PCR mixture (50 µL) contained 1x ammonium buffer (Bioline, Luckenwalde, Germany), 5 mM MgCl₂ (Bioline), 0.2 mM dNTP mix (SIGMA-ALDRICH), 15–30 ng of extracted DNA (quantified by a ND-1000 Nanodrop, PEQLAB Biotechnology, Erlangen, Germany), 1.25 units of BioTaq DNA polymerase (Bioline), and 500 nM of each primer. PCR was performed in a SensoQuest LabCycler (SensoQuest, Göttingen, Germany), with an initial denaturation at 94 °C for 3 min,

followed by 30 cycles of 94 °C for 1 min, 50 °C for 25 s, 72 °C for 2 min, and a final extension at 72 °C for 5 min. PCR products were checked on a 1% agarose gel. Bands of the correct size were excised and extracted using the QIAquick Gel Extraction Kit (QIAGEN). 16S rDNA fragments were cloned into pCR®4-TOPO® plasmids used to transform chemically competent One-Shot® MACH1™ *Escherichia coli* cells (TOPO TA Cloning® Kit, Invitrogen, Darmstadt, Germany) according to the manufacturer's instructions. Colonies containing inserts were isolated by streak-planting onto LB agar mixed with 50 µg/mL ampicillin. Plasmid inserts were screened using colony PCR with M13F forward and M13R reverse primers. Colony PCR products of the correct size were purified using the QIAquick PCR purification kit (QIAGEN) and sequenced at the Göttingen Center of Molecular Biology using the plasmid-specific primers T3 and T7. Sequences were assembled using CodonCode Aligner version 3.7.1.1 (CodonCode Corporation, Dedham, MA, USA) and manually checked for ambiguities. They were screened for chimeras using Bellerophon version 3 (Huber *et al.*, 2004). Putative chimeras were excluded from subsequent analyses. A total of 144 non-chimeric 16S rDNA sequences were submitted to GenBank (accession numbers JN983537–JN983680).

Phylogenetic analysis of 16S rDNA clone library sequences

Sequences obtained from clone libraries were compared to sequences in the public GenBank database using nucleotide BLAST (Altschul *et al.*, 1990). 78 sequences were found to be closely related to sequences of cultivated *Thiothrix* species and to sequences previously obtained from *N. ictus* and *Thiothrix*-dominated microbial mats in Frasassi. They were used for phylogenetic analyses together with 47 closely related *Thiothrix* sequences downloaded from GenBank. All sequences were aligned using the MAFFT version 6 multiple sequence alignment tool (Kato and Toh, 2010) implemented with the Q-INS-I strategy for consideration of RNA secondary structure (Kato and Toh, 2008). The alignment was manually refined, and a 50% consensus filter was applied in MOTHUR (Schloss *et al.*, 2009), resulting in 1369 nucleotide positions used for phylogenetic analysis. jModelTest version 0.1.1 (Posada, 2008) was used to determine the best-suited nucleotide model among 88 possible models

following the Bayesian Information Criterion. The selected model (GTR+G) was used to build a Maximum Likelihood (ML) phylogenetic tree (1000 bootstrap replicates) using PhyML 3.0 (Guindon and Gascuel, 2003). The ML tree was rooted with an epibiont clone sequence from the hydrothermal vent galatheid crab *Shinkaia crosnieri* (GenBank accession number AB476284; Watsuji *et al.*, 2010). In addition, Neighbor-Joining (NJ) bootstrap values for all nodes were calculated based on the same alignment using the BioNJ algorithm (Kimura 2-parameter model; 1000 bootstrap replicates) implemented in SeaView version 4 (Gouy *et al.*, 2010). The resulting *Thiothrix* phylogenetic tree showed that most of the *Niphargus* epibiont sequences clustered into three distinct clades, which were named T1, T2, and T3 (Figure 1.2).

Fluorescence *In Situ* Hybridization (FISH)

Based on sequences obtained from the 16S rDNA clone libraries, oligonucleotide FISH probes specific to *Thiothrix* clades T1–T3 (Figure 1.2) were designed and evaluated as described in Hugenholtz *et al.* (2002). Using PRIMROSE (Ashelford *et al.*, 2002), the probes were checked against other publicly available sequences, especially those associated with Frasassi. Helper probes (Fuchs *et al.*, 2000) served for increasing the chance of hybridization to poorly accessible target sites within the 16S rRNA, and competitor probes (Hugenholtz *et al.*, 2002) were designed to prevent probe binding to other, non-target *Thiothrix* ectosymbiont sequences. All probes used in this study (see Supplementary Table S1.2 for a list of corresponding sequences) were synthesized at Eurofins MWG Operon (Ebersberg, Germany).

FISH probes specific to T1–T3, fluorescently labeled with either fluorescein isothiocyanate (FITC) or cyanine 3 (cy3), were mixed with equimolar amounts of unlabeled competitor and helper probes to make the probe mixes NSPT1mix–NSPT3mix. To determine optimal hybridization stringencies, a FITC-labeled competitor probe with one mismatch to the respective target sequence was added to each probe mix containing a cy3-labeled clade-specific probe. Formamide concentrations were increased stepwise until the green fluorescence signal from the competitor probe disappeared and only the red signal from the clade-specific probe was detected.

33 *Niphargus* individuals and eight microbial mat samples collected between 2008 and 2011 from nine different Frasassi cave locations were examined using the T1–T3 clade-specific FISH probes. *Niphargus* and microbial mat samples for FISH were fixed in 4% paraformaldehyde for 3 h at 4 °C, transferred to a 1:1 ethanol-PBS solution, and stored at –20 °C until analysis. Several legs of each *Niphargus* individual were dissected, transferred to an eppendorf vial with 100 µL of 1x PBS, and sonicated for 1 minute to release the epibionts. Droplets of bacterial suspensions (epibionts or mat bacteria) were applied onto objective slides, and hybridization was carried out for 1.5 h as described in Amann (1995). Additionally, hybridization of entire *Niphargus* legs was carried out in eppendorf tubes. Since all probe mixes had optimal hybridization stringencies of 45%, two probe mixes could be applied at a time to the same sample. Furthermore, a general bacterial EUBmix probe (Daims *et al.*, 1999) was applied in combination with NSPT1mix, NSPT2mix, and NSPT3mix. Samples were mounted with Citifluor (Agar Scientific, Essex, UK) and examined under a Zeiss Axioplan microscope. Whole *Niphargus* legs subjected to FISH were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA), and confocal epifluorescence micrographs of attached bacteria were collected on a Zeiss 510 Meta laser scanning microscope equipped with argon and helium-neon lasers (488 and 543 nm).

Automated Ribosomal Intergenic Spacer Analysis (ARISA) & 16S-ITS clone library construction

ARISA detects length variations in the hypervariable bacterial internal transcribed spacer (ITS) region (Fisher and Triplett, 1999). 40 *Niphargus* individuals collected in 2008 and 2009 from eight different cave locations were examined using this molecular fingerprinting technique. ARISA-PCR was conducted as described in Meziti *et al.* (2010). All DNA samples were analyzed in triplicate. Preparation for capillary electrophoresis separation and analyses of ARISA profiles were done as described in Boer *et al.* (2009). Bin frames of 2 base pairs (bp) window size and a shift window of 1.4 bp were selected by automatic binning (Ramette, 2009). ARISA triplicate profiles were combined so that only operational taxonomic units (OTUs) occurring in at least two of the three replicates were kept to define the final consensus profiles.

In order to identify OTUs in the ARISA profiles belonging to *Thiothrix* clades T1–T3, 16S-ITS clone libraries of *Niphargus*-associated epibiont communities were constructed. DNA extracted from three individuals of each *Niphargus* species (*N. frasassianus* from cave locations ST, RS, PC; *N. ictus* from cave locations LV, LC, PC; *N. montanarius* from cave location BG) was PCR-amplified using the tailored universal forward primer 520F (modified after Muyzer *et al.*, 1996; complementary to *E. coli* positions 520 to 534 of the 16S rRNA) and the bacterial domain-specific reverse primer ITSReub (Cardinale *et al.*, 2004; complementary to *E. coli* positions 23 to 37 of the 23S rRNA). The PCR mixture (50 μ L) contained 1x PCR buffer (Promega, Madison, WI, USA), 1.5 mM MgCl₂ (Promega), 0.25 mM dNTP mix (Promega), 1.5 mL bovine serum albumine (3 μ g/ μ L), 20–25 ng of extracted DNA (quantified by a ND-1000 Nanodrop, PEQLAB), 2.5 units of GoTaq DNA polymerase (Promega), and 400 nM of each primer. PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, 57 °C for 45 s, 72 °C for 90 s, and a final extension at 72 °C for 5 min.

For each PCR, we used a set of three-nucleotide tags conjugated with the 5' ends of forward and reverse primers in order to use the mark–recapture cloning method (Bierne *et al.*, 2007). PCR products from individuals of the same *Niphargus* species were pooled before cloning, and the 5' tags enabled identification of the *Niphargus* individual from which the respective sequence was obtained. Partial 16S-ITS sequences were assembled and manually checked for ambiguities with CodonCode Aligner version 3.7.1.1, and were submitted to Genbank (accession numbers JQ217431–JQ217456). ITS sequences belonging to *Thiothrix* clades T1–T3 were identified based on the adjoining 16S rDNA partial sequences, and their lengths were determined as distances between the target sites of the ARISA-PCR forward and reverse primers.

Statistical analyses

Taking only the ARISA OTUs corresponding to T1–T3 *Thiothrix* into consideration, pairwise similarities among *Niphargus* samples were calculated based on the Bray-Curtis index of dissimilarity (Legendre and Legendre, 1998). The resulting matrix was used to examine patterns in *Thiothrix* distribution among the three *Niphargus* hosts

via Non-Metric Multidimensional Scaling (NMDS). NMDS places all samples in a two-dimensional coordinate system so that the ranked dissimilarities between the samples are preserved, and a stress function measures how well the original ranked distances fit into the reduced ordination space (Ramette, 2007). Analyses of similarities (ANOSIM) were performed to test for significant differences between predefined groups of samples (here *N. frasassianus*, *N. ictus*, and *N. montanarius*) using 1000 Monte-Carlo permutation tests. The resulting test statistic R indicates the degree of separation, ranging from 0 (no separation) to 1 (complete separation). As multiple comparisons were performed, significant ANOSIM R values were identified at the Bonferroni-corrected level ($p < 0.05/k$, with $k = n(n-1)/2$, k representing the number of pair-wise comparisons between n samples). All analyses were implemented within the statistical R environment (R Development Core Team, 2011) using the vegan package (Oksanen *et al.*, 2011) and custom R scripts (Ramette, 2009).

Nested-PCR assays

PCR primers specific to *Thiothrix* clades T1–T3 (Supplementary Table S1.1) were designed based on the corresponding 16S-ITS sequences and used to screen 40 *Niphargus* individuals collected in 2008 and 2009 from eight different cave locations and all eight microbial mat samples previously investigated with FISH. A nested-PCR approach was used to increase the sensitivity of the screenings (Supplementary Figure S1.1). In a first PCR round, bacterial 16S rDNA and ITS sequences were amplified by using the bacterial domain-specific primers 27F and ITSReub. Using the products of the first PCR as templates, a second PCR round was performed using either the *Thiothrix*-specific forward primer THIO714F or the clade-specific forward primers T2_1246F and T3_841F, as appropriate, in combination with clade-specific ITS reverse primers.

Nested-PCR was also applied to obtain partial 16S sequences of those free-living *Thiothrix* bacteria previously marked by the T2-specific FISH probe NSPT2 and to compare them with T2 sequences in 16S clone libraries of *N. ictus* and *N. montanarius*. Again using products of the first PCR round as templates, a third PCR was performed with Frassasi microbial mat samples collected in 2011 using the bacterial domain-

specific forward primer 27F in combination with the clade T2-specific 16S reverse primer T2_1244R (whose sequence was congruent with that of FISH probe NSPT2).

PCR mixtures (20 μ L) contained 1x ammonium buffer (Bioline), 2 mM MgCl₂ (Bioline), 0.2 mM dNTP mix (Bioline), 2 μ L of DNA extract (5–15 ng/ μ L; for the first PCR) or 2 μ L of first PCR products (for the second and third PCR), 0.5 units of BioTaq DNA polymerase (Bioline), and 500 nM of each primer. PCR cycling conditions were identical with those used for 16S rDNA clone library construction, except for a primer annealing temperature of 56 °C for the second and third PCR rounds. PCR products were checked on a 1% agarose gel, and bands of the expected size were excised and purified using the QIAquick Gel Extraction Kit (QIAGEN). Purified products were sequenced as described above. PCR sequences were compared with T1, T2, and T3 sequences previously obtained from 16S rDNA and 16S-ITS clone libraries and submitted to GenBank (accession numbers JX435482–JX435601).

16S rDNA clone libraries of *N. ictus* did not contain any sequences that clustered within *Thiothrix* clade T3 (Figure 1.2). However, T3 *Thiothrix* were detected on *N. ictus* individuals using FISH, ARISA, as well as PCR screenings followed by sequencing. In order to compare T3 sequences between the three *Niphargus* species, a second phylogenetic tree was constructed using the portion of the 16S rDNA sequences amplified by the T3-clade specific primers (Supplementary Figure S1.2).

1.4 Results and Discussion

Diversity of *Thiothrix* ectosymbionts of Frasassi-dwelling *Niphargus* species

The Frasassi-dwelling *Niphargus* were found to be associated with a bacterial epibiont community dominated by *Thiothrix*. SEM examination revealed that individuals of all three *Niphargus* species harbored filamentous bacteria which were attached via holdfasts to hairs and spines on the hosts' legs and antennae (Figure 1.3). The quantity of filaments varied between individual hosts. While both examined *N. frasassianus* specimens and the only inspected *N. montanarius* individual harbored abundant, long bacterial filaments often arranged in rosettes (Figure 1.3, panels A and C), three out of nine investigated *N. ictus* individuals carried only very few, solitary filaments.

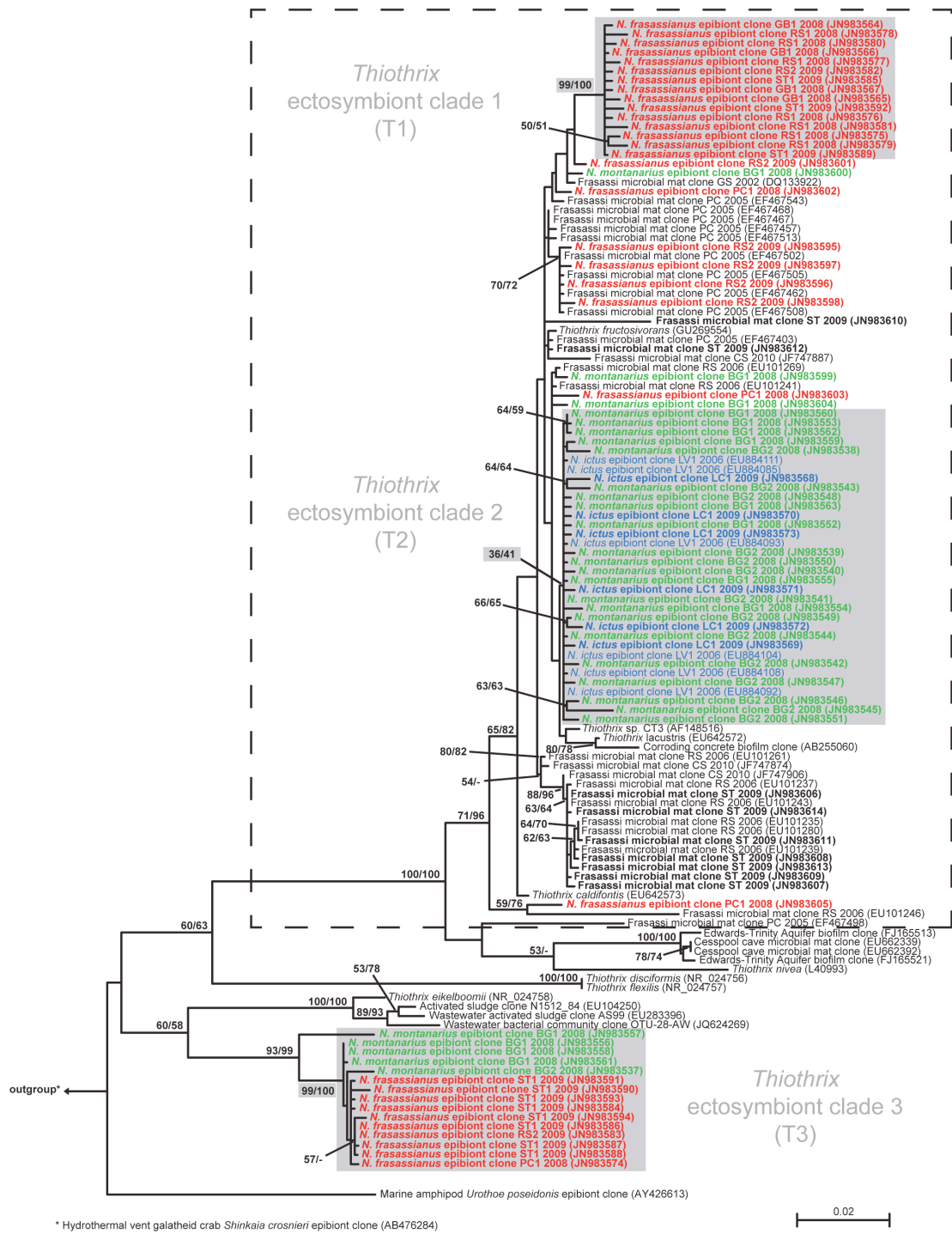


Figure 1.2. 16S rDNA Maximum Likelihood phylogenetic tree of cultivated and uncultivated *Thiothrix* species. Sequences obtained in this study are in bold face, and clone names indicate the sampling location and year (BG = Il Bugianardo, ST = Sorgente del Tunnel, GB = Grotta Bella, LV = Lago Verde, PC = Pozzo dei Cristalli, RS = Ramo Sulfureo, LC = Lago Claudia). Different numbers after cave name abbreviations indicate *Niphargus* individuals collected from the same cave location. *N. ictus* LV 2006 clones are from a previous study (Dattagupta *et al.*, 2009). GenBank accession numbers are given in parentheses. Maximum Likelihood/Neighbor-Joining bootstrap values greater than 50% are listed next to the respective nodes, and the bootstrap value for clade T2 is also indicated. The dashed line encloses those *Thiothrix* sequences obtained from Frasassi microbial mats.

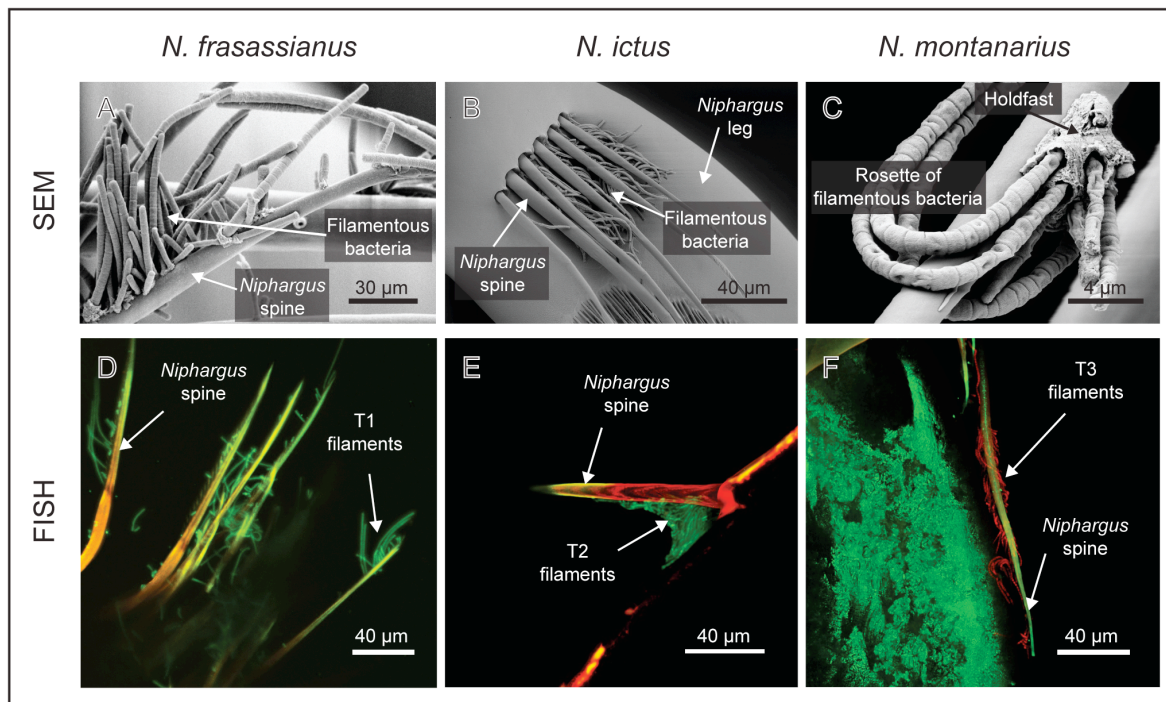


Figure 1.3. SEM and FISH images of *Thiothrix* filaments on Frasassi-dwelling *Niphargus* species. Panels A–C: Scanning electron micrographs (SEM) of bacterial filaments attached to hairs and spines on appendages of the three *Niphargus* species. The arrangement of the filaments in rosettes and their attachment via holdfasts (Panel C) are characteristic features of the bacterial genus *Thiothrix* (Unz and Head, 2005). Panels D–F: Confocal epifluorescence micrographs of *Thiothrix* filaments subjected to Fluorescence *In Situ* Hybridization (FISH) using probes specific to T1–T3 ectosymbiotic clades. Probes NSPT1 and NSPT2, designed to bind to T1 and T2, respectively (Panels D and E), were labeled with the fluorescent dye FITC (in green), T3-specific probe NSPT3 was labeled with cy3 (in red; Panel F).

Eight out of the nine 16S rDNA clone libraries constructed from *Niphargus* individuals contained *Thiothrix* sequences in different percentages (*N. frassianus* from GB 27%, from PC 24%, from RS 70% and 54%, from ST 92%; *N. ictus* from BG 0%, from LC 60%; *N. montanarius* from BG 100% and 100%). A majority of these *Thiothrix* sequences (84%) clustered into three different phylogenetic clades, referred to as T1, T2, and T3 (Figure 1.2). Clade T1, supported by a 99% ML bootstrap value, contained only *Thiothrix* sequences obtained from *N. frassianus*. Clade T2 (bootstrap value 36) was formed by *Thiothrix* sequences from *N. ictus* individuals analyzed in the present as well as in a previous study (Dattagupta *et al.*, 2009) and by sequences obtained from *N. montanarius*. Clade T3 (99% ML bootstrap support) was comprised of *Thiothrix* sequences from *N. montanarius* and *N. frassianus*. T1–T3 were considered to be *Niphargus* symbiont clades, as sequences in these groups were found

consistently in clone libraries from several *Niphargus* individuals collected in 2006, 2008, and 2009 from seven different cave sites.

Some *Thiothrix* sequences from *N. frassianus* and *N. montanarius* clone libraries did not fall within the clades T1–T3, but instead clustered with *Thiothrix* sequences from Frassasi microbial mats (Figure 1.2). Each of these sequences was found either only once or on a single *Niphargus* individual. Moreover, using FISH we found that all filamentous bacteria attached to *Niphargus* appendages bound to one of the T1–T3 clade-specific probes NSPT1–NSPT3 (Figure 1.3, panels D–F). We thus concluded that the additional *Thiothrix* sequences belonged to rare epibionts or to free-living *Thiothrix* that contaminated the *Niphargus* samples during collection. Sequences belonging to other types of bacteria were also obtained in clone libraries from several *N. ictus* and *N. frassianus* individuals (Supplementary Table S1.3). None of these additional non-*Thiothrix* sequences was found consistently on different *Niphargus* of the same species, providing insufficient evidence for them to be regarded as symbionts.

16S rDNA clone libraries suggested that T1 ectosymbionts are solely harbored by *N. frassianus*, T2 by *N. ictus* and *N. montanarius*, and T3 by *N. frassianus* and *N. montanarius* (Figure 1.2). However, only *N. ictus* individuals collected from cave locations LV and LC were used for clone library construction. FISH analyses confirmed the results obtained from the clone libraries, but additionally revealed T3 filaments on *N. ictus* individuals from cave locations BG and PC (Table 1.1). Moreover, ARISA as well as nested-PCR detected the presence of T3 *Thiothrix* on *N. ictus* from all sampled cave locations, including LV and LC (Figure 1.4; Table 1.1). This discrepancy between the results of the different methods might be explained by very low amounts of T3 *Thiothrix* cells on *N. ictus* individuals from LV and LC, which might have been sufficient to be detected by highly sensitive techniques like ARISA and nested-PCR, but insufficient to become represented in 16S rDNA clone libraries or be revealed by FISH. The overall evidence suggests that T3 *Thiothrix* are ectosymbionts of all three Frassasi-dwelling *Niphargus* species.

Chapter 1 - Host-specific *Niphargus-Thiothrix* ectosymbioses

Table 1.1. Results of FISH experiments and nested-PCR assays on *Niphargus* individuals. NSPT1–NSPT3 = *Niphargus* Symbiont Probes specific to *Thiothrix* clades T1–T3. GS = Grotta Sulfurea, ST = Sorgente del Tunnel, GB = Grotta Bella, PC = Pozzo dei Cristalli, RS = Ramo Sulfureo, BG = Il Bugianardo, LV = Lago Verde, LC = Lago Claudia. n.a. = not analyzed.

<i>Niphargus</i> individuals			FISH results				PCR results			
Species	Cave	Year	Individuals analyzed (n)	# of individuals harboring filaments that bound to			Individuals analyzed (n)	# of individuals containing		
				NSPT1	NSPT2	NSPT3		T1	T2	T3
<i>Niphargus frassianus</i>	GS	2008	0	n.a.	n.a.	n.a.	4	4	3	4
		2009	1	1	0	1	0	n.a.	n.a.	n.a.
	ST	2009	2	2	0	2	2	2	2	2
		2010	6	6	0	6	0	n.a.	n.a.	n.a.
	GB	2008	0	n.a.	n.a.	n.a.	3	3	3	3
		2009	1	1	0	1	0	n.a.	n.a.	n.a.
	PC	2008	0	n.a.	n.a.	n.a.	1	1	1	1
		2009	3	3	0	3	2	2	2	2
	RS	2008	0	n.a.	n.a.	n.a.	3	3	3	3
2009		0	n.a.	n.a.	n.a.	4	4	4	4	
<i>Niphargus ictus</i>	BG	2008	0	n.a.	n.a.	n.a.	2	0	2	2
		2011	2	0	2	2	0	n.a.	n.a.	n.a.
	LV	2008	0	n.a.	n.a.	n.a.	4	0	4	4
		2009	1	0	1	0	2	0	2	1
		2010	1	0	1	0	0	n.a.	n.a.	n.a.
		2011	6	0	6	0	0	n.a.	n.a.	n.a.
	PC	2008	0	n.a.	n.a.	n.a.	3	2	3	3
		2009	2	0	2	1	2	2	2	2
	RS	2008	0	n.a.	n.a.	n.a.	2	1	2	2
	LC	2009	0	n.a.	n.a.	n.a.	1	0	1	1
<i>Niphargus montanarius</i>	BG	2008	0	n.a.	n.a.	n.a.	5	2	5	5
		2010	6	0	6	6	0	n.a.	n.a.	n.a.
		2011	1	0	1	1	0	n.a.	n.a.	n.a.

Origins of the *Niphargus-Thiothrix* ectosymbioses

Closest relatives of ectosymbionts T1 and T2 are *Thiothrix* from Frassassi cave microbial mats (Figure 1.2). Thus, it is likely that T1 and T2 symbionts have evolved within the cave system from previously free-living bacteria. Due to poor bootstrap support for nodes connecting clades T1 and T2, it is not possible to say whether the ectosymbionts evolved from a common symbiotic ancestor or from distinct free-living *Thiothrix*. The three Frassassi-dwelling *Niphargus* species probably invaded the cave

system independently, but in a yet unknown order (Flot *et al.*, 2010a). Therefore, it is currently not possible to speculate about the sequence in which T1 and T2 symbionts were acquired by their different hosts.

The T3 clade is distantly related to clades T1 and T2 and to all other Frasassi cave microbial mat sequences. Consequently, T3 ectosymbionts seem to have originated from outside the Frasassi cave system. The three *Niphargus* species investigated in this study are distantly related to each other (Flot *et al.*, 2010a), and each of them harbors T3 symbionts. This suggests that T3 symbionts are either widespread in the genus *Niphargus* or were introduced into Frasassi by one *Niphargus* host and subsequently dispersed over the remaining species inside the caves. Investigations of non-Frasassi *Niphargus* species for *Thiothrix* ectosymbionts are currently underway to evaluate these alternatives.

Our analyses suggest that the *Niphargus-Thiothrix* symbiosis has evolved independently at least two times. Symbioses with T1 and T2 may have been initiated within the last one million years, during which sulfidic conditions within the Frasassi caves have been established (Dattagupta *et al.*, 2009), whereas the association with T3 is likely more ancient.

Thiothrix ectosymbiont transmission mode

FISH with oligonucleotide probes specific to T1–T3 was used to examine whether significant free-living populations of the ectosymbionts were present in Frasassi cave microbial mats. These analyses revealed that T1 filaments were nearly absent from the mats (Figure 1.5), except for two short filaments (both <10 µm) observed in two samples. Free-living *Thiothrix* filaments tend to be several 100 micrometers in length, while ectosymbiont filaments on *Niphargus* appear to be “groomed” to lengths between 30 and 100 µm (Figure 1.3). It is thus likely that the short T1 filaments detected within the mat samples were shed ectosymbionts rather than steady members of the microbial mat community. T2-specific probe NSPT2 bound to few filaments in mats collected before or in the year 2009 (Dattagupta *et al.*, 2009; Figure 1.5), but to considerably more filaments in mats collected in 2011. T3 filaments were not detected in any of the eight Frasassi microbial mat samples analyzed by FISH (Figure 1.5).

Bacterial symbionts can either be transmitted horizontally, where they get repeatedly recruited from the host's environment, or vertically, where they are passed down from one host generation to the next (Bright and Bulgheresi, 2010). Considering the dearth of T1 and T3 free-living counterparts in Frasassi microbial mats, horizontal transmission through the perpetual reacquisition of these symbionts from the mats appears doubtful. In the *Thiothrix* phylogenetic tree (Figure 1.2), a relatively long branch separates clade T1 from Frasassi microbial mat sequences, and the T3 clade is very distinct from all sequences obtained from Frasassi. Both clades are also strongly supported by ML bootstrap values of 99%. This is consistent with the increased genetic isolation and homogeneity that accompanies vertical symbiont transmission (Moran, 1996; Moran and Plague, 2004; Bright and Bulgheresi, 2010).

FISH revealed a significant free-living population of T2 in Frasassi cave microbial mats collected in 2011 (Figure 1.5A). However, 16S rDNA sequences amplified from the 2011 mat samples using T2-specific PCR primers had a consistent one-base difference to all sequences derived from *N. ictus* and *N. montanarius* (Figure 1.5B). From this it follows that T2 ectosymbionts of *N. ictus* and *N. montanarius* are very closely related to but nevertheless distinct from the filamentous mat bacteria marked by probe NSPT2. This indicates a lack of interchange between the free-living and ectosymbiotic T2 communities, suggesting that horizontal transmission is not prevalent.

16S rDNA sequences of T2 symbionts derived from *N. ictus* and *N. montanarius* are indistinguishable. This suggests that these symbionts freely alternate between their respective *Niphargus* hosts. In the case of T3, three distinct types of 16S rDNA sequences were obtained: one derived from only one *N. montanarius* individual, a second from several individuals of all three *Niphargus* species, and a third from several individuals of *N. ictus* and one *N. montanarius* individual (Supplementary Figure S1.2). It is possible that the three subtypes of T3 were introduced into Frasassi by the three *Niphargus* species, and two of them were subsequently exchanged among different hosts. Taken together, the evidence indicates that T2 and T3 symbionts are transmitted among different hosts using a combination of intra- and interspecific inoculations, whereas T1 symbionts are transmitted exclusively among *N. frasassianus*.

Vertical transmission has been commonly reported for endosymbioses where symbionts are passed down through the host female germ line (Bright and Bulgheresi, 2010). A “pseudo-vertical” transmission mode was previously suggested for *N. ictus*, whereby symbionts are transmitted externally onto eggs or juveniles carried in the female’s brood-pouch (Dattagupta *et al.*, 2009). The *Niphargus* sample set used for ARISA (Figure 1.4) included a *Niphargus* baby (“RS08_223 (young)”) that had been removed from the brood pouch of the mother animal (“RS08_223 (mother)”) just before DNA extraction. ARISA results revealed that the juvenile individual, like its mother, harbored T2 and T3 ectosymbionts on its appendages.

Thiothrix bacteria can relocate themselves through gonidia, which are motile dispersal cells produced from the apex of the filaments (Larkin and Shinabarger, 1983; Chernousova *et al.*, 2009). Nested-PCR assays detected T1, T2, and T3 in many Frasassi microbial mats, including samples where FISH had indicated an absence of full-grown filaments (Figure 1.5). The highly sensitive PCR analyses presumably detected *Thiothrix* gonidia that are present in Frasassi cave waters. These gonidia may be the means by which T1–T3 ectosymbionts are exchanged among *Niphargus* hosts living in various cave locations throughout Frasassi.

Host-specific *Thiothrix* distribution patterns

ARISA served to identify compositional differences in the *Thiothrix* communities associated with the three *Niphargus* species. Determining the ITS lengths of the *Thiothrix* symbionts from 16S-ITS clone libraries allowed us to assign individual OTUs in the ARISA consensus profiles to T1, T2, and T3. Symbiont ITS lengths were as follows: 647 bp (T1), 651, 652 bp (T2), and 661, 662 bp (T3). Taking only the ARISA OTUs corresponding to these five ITS sequence lengths into account for NMDS analysis, calculated pairwise similarities confirmed that the relative distribution patterns of *Thiothrix* symbionts on the three *Niphargus* species were host species-specific (Figure 1.4). Except for two outliers (*N. ictus* BG08_203 and *N. montanarius* BG08_005, which were both dominated by the 662bp T3 OTU), all analyzed *Niphargus* samples clustered into three different groups according to their respective host species. This was the case even for *Niphargus* individuals of two different species that

co-occurred and were collected simultaneously at the same cave locations (BG, PC, RS). *N. ictus* and *N. frasassianus* inhabit several different cave sites where the dominant microbial mat type varies based on the cave water geochemistry and flow regime (Figure 1.1). However, neither sampling location nor collection year had a major influence on the relative *Thiothrix* ectosymbiont distribution patterns on these hosts. Despite the two outliers, ANOSIM statistics (R and p values) confirmed clear separation with high significance between the three *Niphargus* species. It is however important to note that the sample size of *N. montanarius* (n=5) was much smaller than that of *N. ictus* (n=16) and *N. frasassianus* (n=19). Unequal sample sizes lower the meaningfulness of ANOSIM, but were unavoidable in our study due to the low availability of *N. montanarius* individuals.

T1 and T3 OTUs were abundant on *N. frasassianus*, whereas *N. ictus* and *N. montanarius* were mainly associated with T2 and T3. This is in close agreement with our FISH results (Table 1.1). The clear separation between the three *Niphargus* species in the NMDS plot generated from ARISA (Figure 1.4) was obtained using a Bray-Curtis index of dissimilarity for the calculations, which considers both presence/absence and relative abundance of *Thiothrix* OTUs. Using a Jaccard index instead, which takes only OTU presence/absence into account, resulted in non-significant ANOSIM values and poor separation between the three groups in the NMDS plot. Close examination showed that T2 ARISA OTUs were also occasionally detected on *N. frasassianus*, and T1 OTUs on *N. ictus* and *N. montanarius*. Thus, the ARISA results indicate that T1–T3 *Thiothrix* ectosymbionts can colonize all three *Niphargus* hosts, but their abundances are strongly influenced by the identity of the host species they are associated with. This is consistent with the comparison between FISH and PCR results, where FISH shows a host-species specific distribution pattern, whereas nested-PCR assays detected T1–T3 sequences on all three *Niphargus* species (Table 1.1). While the highly sensitive nested-PCR approach might trace gonidia attached to *Niphargus* exoskeletons, FISH would only reveal full-grown *Thiothrix* filaments. Taken together, our analyses suggest that T1–T3 ectosymbiont gonidia can attach to exoskeletons of all three *Niphargus* species. However, only T1 and T3 filaments develop successfully on *N. frasassianus*, whereas T2 and T3 flourish on *N. ictus* and *N. montanarius*, with T3 dominating *N. montanarius* ectosymbiont communities.

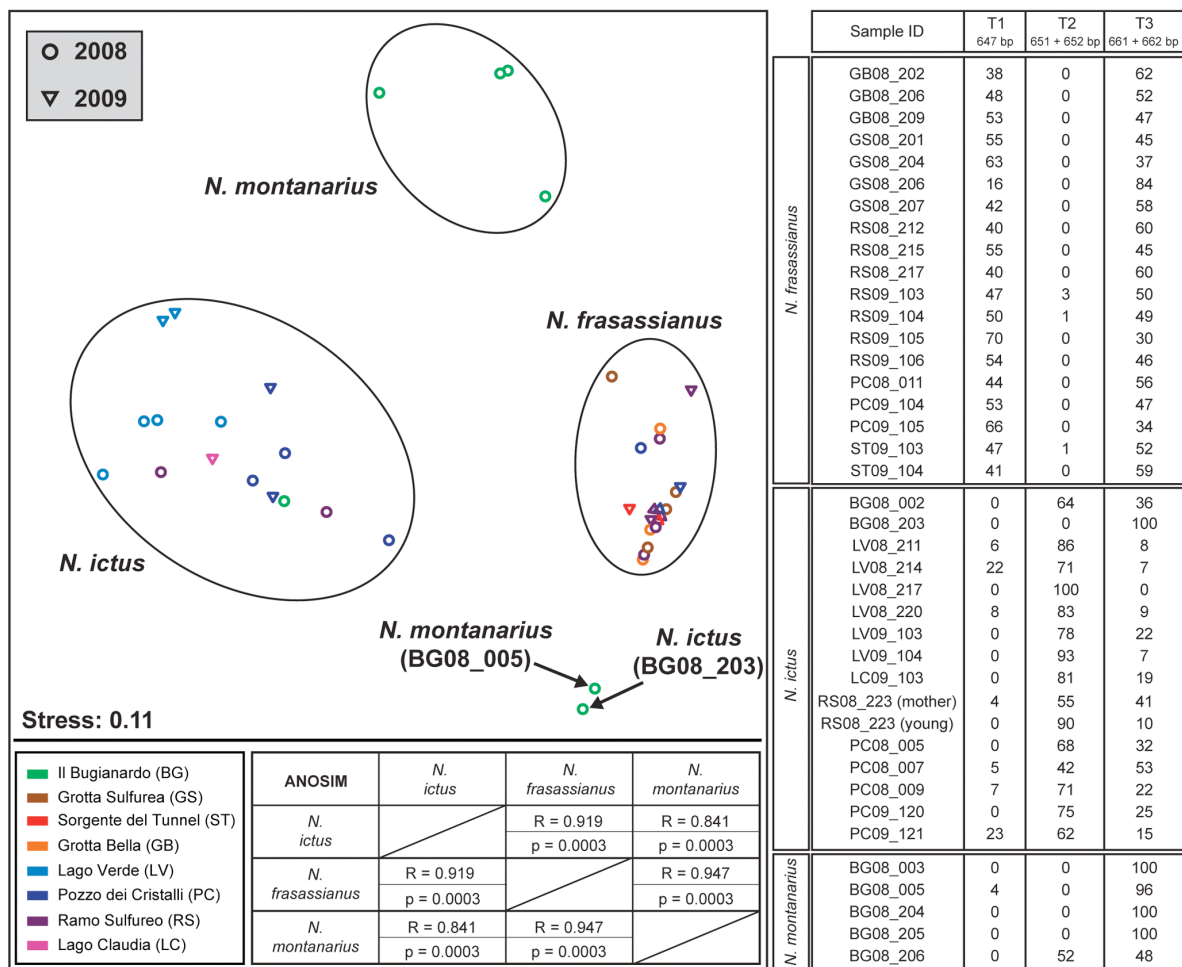


Figure 1.4. Graphical and tabular presentation of ARISA results. Left: Non-metric multidimensional scaling (NMDS) plot, in which each data point represents the *Thiothrix* ectosymbiont community structure associated with one *Niphargus* individual. Circles represent samples collected in May–June 2008 and triangles those collected in May–June 2009 from one of the eight Frasassi cave locations represented by different colors. Pairwise similarities among the *Thiothrix* ectosymbionts of *N. frassassianus*, *N. ictus*, and *N. montanarius* show that their community structures on the three host species are clearly distinct (overall ANOSIM R value: 0.894, overall Bonferroni-corrected p-value: 0.0001). Right: The table shows relative proportions (% among each other) of OTUs assigned to *Thiothrix* symbionts T1–T3 in ARISA consensus profiles of *Niphargus*-associated epibiont communities. Sample IDs indicate the sampling location and year (GB = Grotta Bella, GS = Grotta Sulfurea, RS = Ramo Sulfureo, PC = Pozzo dei Cristalli, ST = Sorgente del Tunnel, BG = Il Bugianardo, LV = Lago Verde, LC = Lago Claudia; for example, GB08_202 indicates that the sample with internal number 202 was collected from cave location GB in 2008).

Chitin, the major component of *Niphargus* exoskeletons, is a common binding motif for many invertebrate-microbe associations (Chaston and Goodrich-Blair, 2010). *Thiothrix* belonging to the clades T1, T2, and T3 all appear to have the ability and the preference to colonize the chitinous surfaces of *Niphargus* amphipods, but their host-specific distribution pattern is likely caused by factors controlled by each *Niphargus* species.

A)

Microbial mat samples		FISH results			PCR results		
Cave location	Collection year	~% filamentous bacteria marked by NSPT1	NSPT2	NSPT3	Detection of		
					T1	T2	T3
Grotta Sulfurea (GS)	2009	0	<1	0	√	√	√
Sorgente del Tunnel (ST)	2009	0	5	0		√	
Grotta Bella (GB)	2009	0	<1	0		√	
Pozzo dei Cristalli (PC)	2009	<<1	<1	0	√	√	
Ramo Sulfureo (RS)	2009	0	<1	0		√	
Cave Spring (CS)	2011	<<1	30	0	√	√	√
Grotta Bella (GB)	2011	0	30	0		√	
Ramo Sulfureo (RS)	2011	0	30	0		√	

B)

T2 detected in microbial mat CS 2011	... TGG GCG C AAG CCT ...
T2 detected in microbial mat GB 2011	... TGG GCG C AAG CCT ...
T2 detected in microbial mat RS 2011	... TGG GCG C AAG CCT ...
T2 epibiont clones <i>N. montanarius</i> (n=22)	... TGG GCG A AAG CCT ...
T2 epibiont clones <i>N. ictus</i> (n=12)	... TGG GCG A AAG CCT ...

Figure 1.5. Results of FISH and PCR analyses of Frasassi microbial mats. (A) NSPT1–NSPT3 = *Niphargus* Symbiont Probes specific to T1–T3. √ = PCR product of expected size verified by sequencing to belong to clades T1, T2, or T3. (B) Consistent one-base difference between T2 16S rDNA sequences derived from microbial mats and *Niphargus* samples. n = Number of clones. Numbers below the sequences refer to 16S rRNA nucleotide positions according to *Escherichia coli* numbering (Brosius *et al.*, 1981). Microbial mat T2 sequences were obtained by sequencing PCR products using T2-specific primers, whereas T2 epibiont sequences were obtained from 16S rDNA clone libraries of *Niphargus*.

Selection of particular ectosymbiont clades may be mediated by lectins secreted on the *Niphargus* cuticle. Such a mechanism has previously been shown to enable differential ectosymbiont acquisition by nematodes dwelling in sulfidic marine sediments (Bulgheresi *et al.*, 2006, 2011). Another possibility is that the three *Thiothrix* ectosymbionts have different tolerances and requirements for sulfide and oxygen. Thus, their respective prevalences on different *Niphargus* species may result from their adaptation to the distinct locomotion behaviors and microhabitat preferences of their hosts. *N. ictus* is a swimming species that prefers stagnant, stratified water bodies. It travels between oxygenated top layers and sulfidic bottom zones and thereby exposes its ectosymbionts to alternating redox conditions (Dattagupta *et al.*, 2009; Flot *et al.*, 2010a). *N. frasassianus* is a poor swimmer and

favors shallow, turbulent streams, where it crawls among sulfide-rich sediments and microbial mats (Flot *et al.*, 2010a). Thus, its ectosymbionts are continuously and simultaneously provided with sulfide and oxygen. *N. montanarius* is found exclusively in cave location BG (Flot *et al.*, 2010a), which is a non-sulfidic pool (Figure 1.1). We are currently examining the metabolic capabilities of the three *Thiothrix* ectosymbionts to infer the benefits that each of them may derive from its particular 'hitch-hiking' lifestyle.

1.5 Conclusion

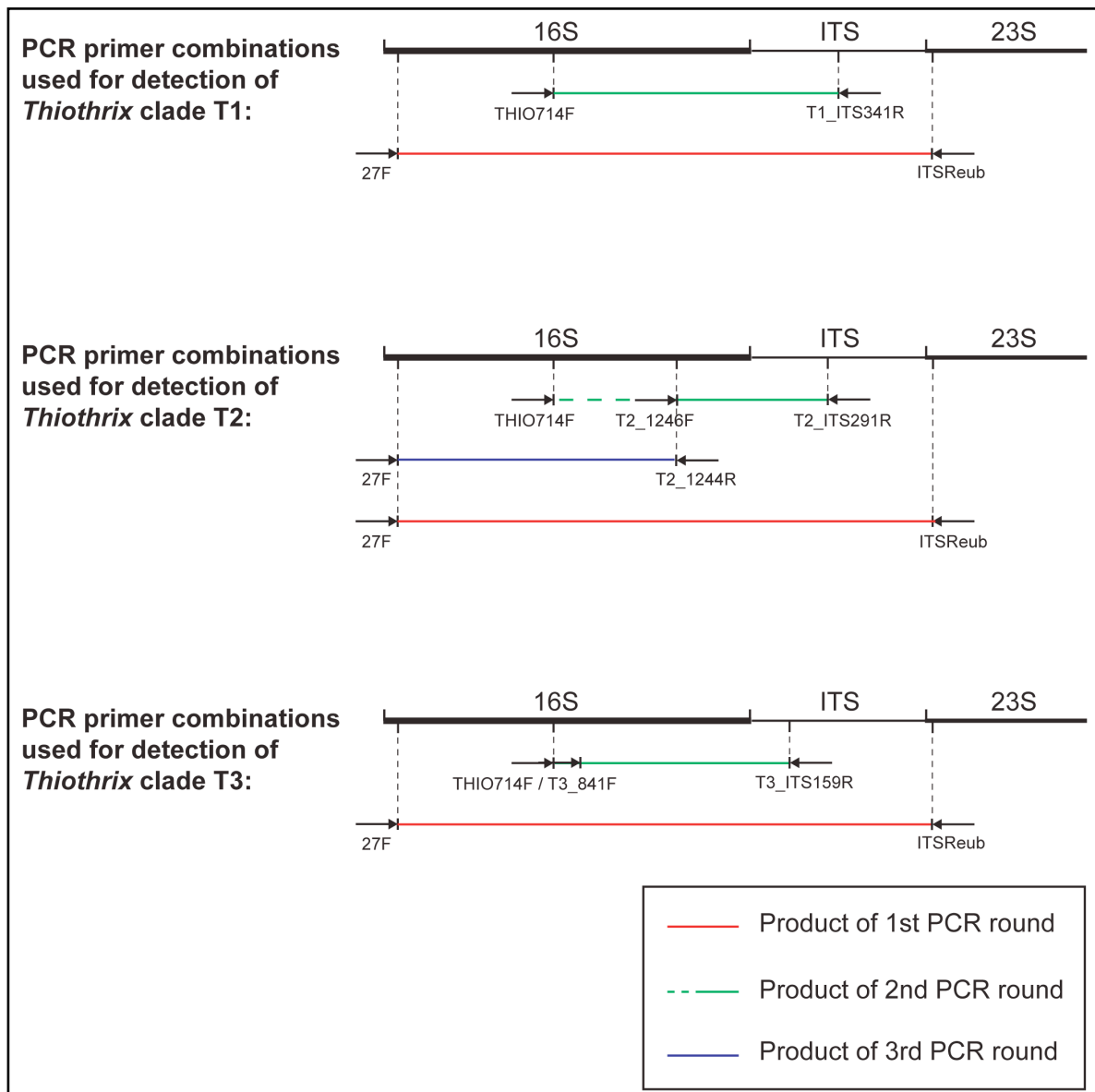
While symbioses between invertebrates and sulfur-oxidizing bacteria have been extensively studied in the marine environment (Ott *et al.*, 2004; Dubilier *et al.*, 2008), the first example from a freshwater ecosystem involving *Niphargus* amphipods was discovered only recently (Dattagupta *et al.*, 2009). In this study, we found that three *Niphargus* species living in the sulfide-rich cave system of Frasassi are ectosymbiotic with filamentous *Thiothrix* ectosymbionts of three different clades. The genus *Niphargus* contains over 300 species distributed throughout Europe (Fišer *et al.*, 2008; Väinölä *et al.*, 2008), some of which are found in other sulfidic locations, such as Movile cave in Romania (Sarbu *et al.*, 1996). *Niphargus-Thiothrix* ectosymbioses are thus potentially widespread in the subterranean realm and warrant further investigation.

1.6 Acknowledgements

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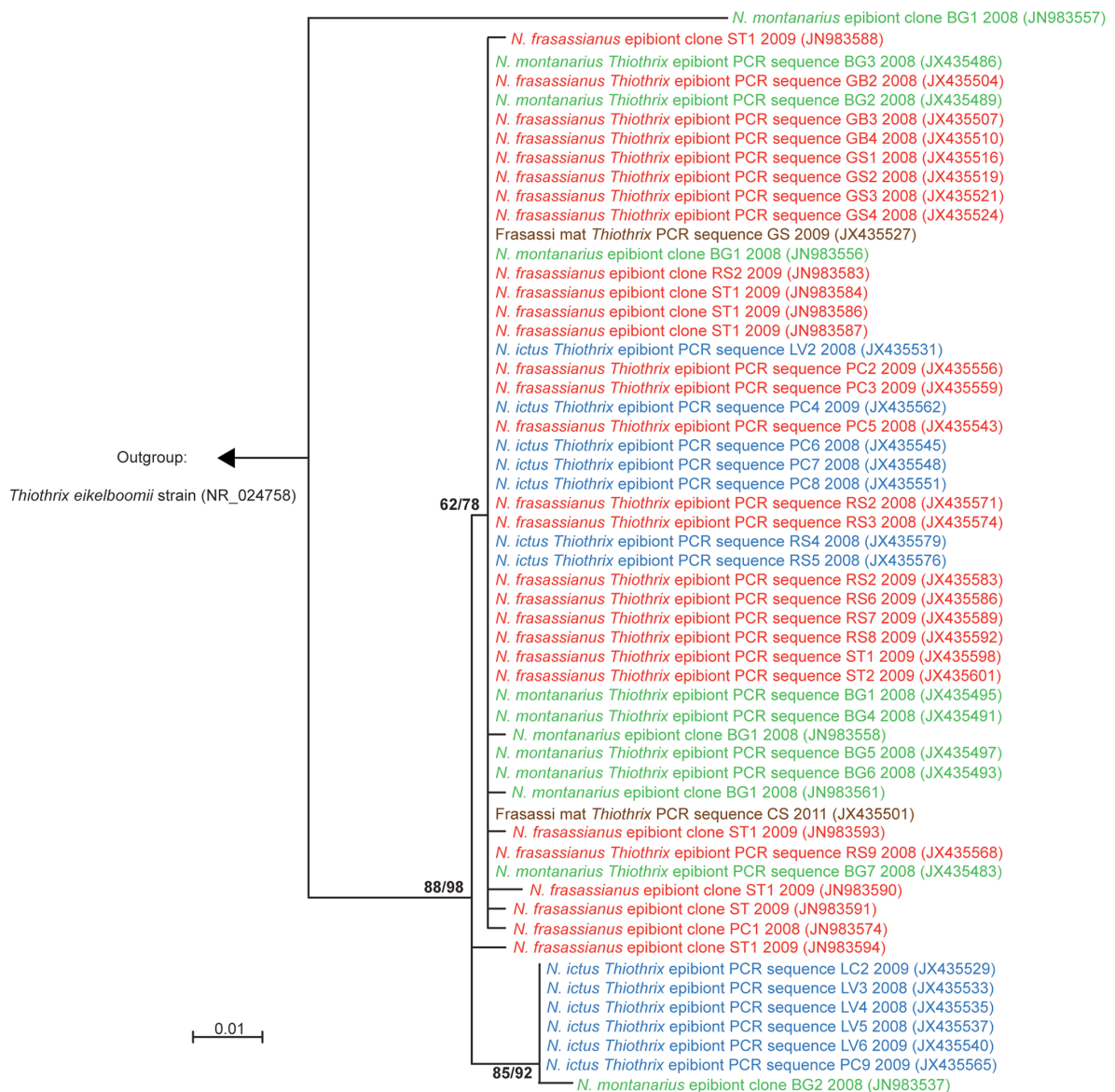
the German Initiative of Excellence and by the Max Planck Society. Fieldwork was financially supported by the National Geographic Committee for Research & Exploration (grant 8387-08 to SD). This is contribution number 103 from the Courant Research Centre Geobiology of the University of Göttingen.

1.7 Supplementary Material



Supplementary Figure S1.1. 16S rDNA and ITS binding sites of *Thiothrix* clade-specific PCR primers.

Chapter 1 - Host-specific *Niphargus-Thiothrix* ectosymbioses



Supplementary Figure S1.2. 16S Maximum Likelihood phylogenetic tree of *Thiothrix* clade T3, including all sequences obtained from 16S clone libraries and nested-PCR assays. Colors mark the different sources from which the sequences were obtained (red = *N. frasassianus*, blue = *N. ictus*, green = *N. montanarius*, brown = Frasassi microbial mats). Clone and sequence names indicate the sampling location and year (BG = Il Bugianardo, GS = Grotta Sulfurea, CS = Cave Spring, ST = Sorgente del Tunnel, GB = Grotta Bella, LV = Lago Verde, PC = Pozzo dei Cristalli, RS = Ramo Sulfureo, LC = Lago Claudia). Different numbers after cave name abbreviations indicate different *Niphargus* individuals collected from the same cave location (cf. Figure 1.2). GenBank accession numbers are given in parentheses. Maximum Likelihood/Neighbor-Joining bootstrap values greater than 50% are listed next to the respective nodes.

Clade T3 phylogenetic tree construction (Supplementary Figure S1.2)

16S portions (~600 bp fragments) of 41 T3 sequences obtained from PCR screenings of *Niphargus* individuals and Frasassi microbial mat samples were aligned with all T3 sequences contained in *Niphargus* 16S clone libraries using the MAFFT version 6 multiple sequence alignment tool (Kato and Toh, 2010) implemented with the Q-INS-I strategy for consideration of RNA secondary structure (Kato and Toh, 2008). The alignment was manually refined, and a 50% consensus filter was applied in MOTHUR (Schloss *et al.*, 2009), resulting in 579 nucleotide positions used for phylogenetic analysis. jModelTest version 0.1.1 (Posada, 2008) was used to determine the best-suited nucleotide model among 88 possible models following the Bayesian Information Criterion. The selected model (TIM3+I) was used to build a Maximum Likelihood (ML) phylogenetic tree (1000 bootstrap replicates) using PhyML 3.0 (Guindon and Gascuel, 2003). The ML tree was rooted using a *Thiothrix eikelboomii* strain sequence downloaded from the GenBank database (accession number NR_024758). In addition, Neighbor-Joining (NJ) bootstrap values for all nodes were calculated based on the same alignment using the BioNJ algorithm (Kimura 2-parameter model; 1000 bootstrap replicates) implemented in SeaView version 4 (Gouy *et al.*, 2010).

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Supplementary Table S1.1. List of PCR primers used in this study.

Primer ¹	Target(s)/Application	Sequence (5' to 3')	Target site ²	Reference
27F	Domain bacteria	AGA GTT TGA TCC TGG CTC AG	16S (8–27)	Weisburg <i>et al.</i> (1991)
1492R	Universal	GGT TAC CTT GTT ACG ACT T	16S (1492–1510)	Weisburg <i>et al.</i> (1991)
M13F	pCR®4-TOPO® plasmids	GTT GTA AAA CGA CGG CCA GT	within plasmid	Invitrogen
M13R	pCR®4-TOPO® plasmids	CAG GAA ACA GCT ATG ACC	within plasmid	Invitrogen
T3	pCR®4-TOPO® plasmids	ATT AAC CCT CAC TAA AGG GA	within plasmid	Invitrogen
T7	pCR®4-TOPO® plasmids	TAA TAC GAC TCA CTA TAG GG	within plasmid	Invitrogen
ITSF	Universal	GTC GTA ACA AGG TAG CCG TA	16S (1494–1513)	Cardinale <i>et al.</i> (2004)
ITSReub	Domain bacteria	GCC AAG GCA TCC ACC	23S (23–37)	Cardinale <i>et al.</i> (2004)
534F	Universal	AGC AGC CGC GGT AAT	16S (520–534)	This study
THIO714F	<i>Thiothrix</i>	ATG CAT AGA GAT CGG AAG G	16S (696–714)	This study
T2_1246F	<i>Thiothrix</i> clade T2	GTA CAG AGG GAA GCA AGA	16S (1246–1263)	This study
T3_841F	<i>Thiothrix</i> clade T3	ATA TAA GAG GTC GGT GGT G	16S (841–859)	This study
T2_1244R	<i>Thiothrix</i> clade T2	TCT TGC TTC CCT CTG TAC CA	16S (1244–1263)	This study
T1_ITS341R	<i>Thiothrix</i> clade T1	CAT TTA TAT GGA ACA GGG TTC G	ITS (341–362)	This study
T2_ITS291R	<i>Thiothrix</i> clade T2	TGG TGT ATT AGC AAG CTA T	ITS (291–309)	This study
T3_ITS159R	<i>Thiothrix</i> clade T3	GCT GGA TGG CAA TTG CTC	ITS (159–176)	This study

¹ F and R suffixes indicate Forward and Reverse primers

² rRNA position according to *Escherichia coli* numbering (Brosius *et al.*, 1980, 1981) and ITS position in *Thiothrix* clade, respectively

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Supplementary Table S1.2. List of FISH probes used in this study.

Probe	Target(s)/Application	Sequence (5' to 3')	Target site ⁵	Reference
EUB338 ¹	Most bacteria	GCT GCC TCC CGT AGG AGT	338–355	Amann <i>et al.</i> (1990)
EUB338-II ¹	Planctomycetales	GCA GCC ACC CGT AGG TGT	338–355	Daims <i>et al.</i> (1999)
EUB338-III ¹	Verrucomicrobiales	GCT GCC ACC CGT AGG TGT	338–355	Daims <i>et al.</i> (1999)
NSPT1 ²	<i>Thiothrix</i> clade T1	CTC CCA GTA TCG GAT GCA GTT	629–649	This study
NST1cP-A ²	Competitor probe, specific to <i>Thiothrix</i> clade T2	CTA CCA GTA TCA GAT GCA GTT	629–649	This study
NST1cP-B ²	Competitor probe, specific to <i>Thiothrix</i> clade T3	TTC CCA GTA TCG GAT GCA GTT	629–649	This study
NST1cP-C ²	Competitor probe, specific to <i>Thiothrix</i> clade T3	TCC CCA GTA TCG GAT GCA GTT	629–649	This study
NST1cP-D ²	Competitor probe, specific to <i>Thiothrix</i> clade T3	TTC CCA GTA TCG GGT GCA GTT	629–649	This study
NST1hP-A ²	Helper probe	GAT TTC ACA TCC GAC TTA AAA AAC CG	586–611	This study
NST1hP-B ²	Helper probe	CTC TCC TCT CCC ACA CTC TAG T	650–671	This study
NST1hP-C ²	Helper probe	CCC AGG TTG AGC CCG GG	612–628	This study
NSPT2 ³	<i>Thiothrix</i> clade T2	TCT TGC TTC CCT CTG TAC CAC C	1242–1263	This study
NST2cP-B ³	Competitor probe, specific to <i>Thiothrix</i> clade T1	TCT TGC CTC CCT CTG TAC TGC C	1242–1263	This study
NST2cP-C ³	Competitor probe, specific to <i>Thiothrix</i> clade T1	TAT TGC AGC TCT CTG TAC GCC C	1242–1263	This study
NST2cP-D ³	Competitor probe, specific to <i>Thiothrix</i> clade T3	TCT TGC GTC CCT CTG TAA CAA C	1242–1263	This study
NST2cP-E ³	Competitor probe, specific to other bacteria	TCT TGC TTC CCT CTG TAC TGC	1243–1263	This study
NST2hP-A ³	Helper probe	ATT TGC TCC ACC TCG CGG	1264–1281	This study
NST2hP-B ³	Helper probe	GAC TAC GGA TGG CTT TCT GGG	1282–1302	This study
NSPT3 ⁴	<i>Thiothrix</i> clade T3	TGC ACC ACC GAC CTC TTA TAT	841–860	This study
NST3cP-B ⁴	Competitor probe, specific to <i>Thiothrix</i> clades T1 and T2	TCC ACC ACC AAA CCC TAA AGC	841–860	This study
NST3hP-A ⁴	Helper probe	GAA GCC GAC GGC TAG TTG ACA	820–840	This study
NST3hP-B ⁴	Helper probe	CGG TCA ACT TAA TGC GTT AGC	861–881	This study

¹ Combined in equimolar amounts to make EUBmix (0–50% formamide)

² Combined in equimolar amounts to make NSPT1mix (45% formamide)

³ Combined in equimolar amounts to make NSPT2mix (45% formamide)

⁴ Combined in equimolar amounts to make NSPT3mix (45% formamide)

⁵ rRNA position according to *Escherichia coli* numbering (Brosius *et al.*, 1981)

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Supplementary Table S1.3. List of non-*Thiothrix* sequences obtained from 16S rDNA clone libraries of *Niphargus*-associated epibionts.

Host species	Sampling location, year	Clone # (GenBank accession No.)	Top blastn hit / source (GenBank accession No.)	Query coverage*	Maximum identity
<i>N. icitus</i>	Il Bugiamardo (BG), 2008	10 (JN983615)	Uncultured bacterium clone reservoir-27 / Dianchi Lake bacterioplankton community (JF697408.1)	100%	93%
		11 (JN983616)	<i>Propionibacterium acnes</i> (AB573714.1)	100%	99%
		13 (JN983617)	<i>Candidatus Hepatoplasma crinochetorum</i> clone 59 / terrestrial isopod gut symbiont (AY500250.1)	100%	87%
		14 (JN983618)	Uncultured <i>Staphylococcus</i> sp. clone EHFS1_S09d / European clean room environments (EU071495.1)	99%	99%
		16 (JN983619)	Uncultured bacterium clone Dianchi-29 / Dianchi Lakeshore polluted water microbial community (HQ324875.1)	100%	96%
		17 (JN983620)	Uncultured Bacteroidetes bacterium clone UMAB-cl-134 / Antarctic Peninsula microbial community (FR749759.1)	100%	95%
		19 (JN983621)	Uncultured Pseudomonadales bacterium clone LCM020 / bacterial colonization on adult black fly (JF733452.1)	100%	94%
		20 (JN983622)	Uncultured bacterium clone reservoir-27 / Dianchi Lake bacterioplankton community (JF697408.1)	100%	94%
		21 (JN983623)	<i>Fangia hongkongensis</i> strain UST040201-002 / coastal seawater of Hongkong (NR_041041.1)	99%	82%
		23 (JN983624)	Uncultured bacterium clone lka65 / Frasassi microbial mat community (EF467563.1)	100%	99%
		25 (JN983625)	Uncultured bacterium clone DLN-150 / fecal microbiota in gayal (FJ848446.1)	100%	97%
		26 (JN983626)	Uncultured bacterium clone reservoir-27 / Dianchi Lake bacterioplankton community (JF697408.1)	100%	94%
		28 (JN983627)	<i>Candidatus Hepatoplasma crinochetorum</i> clone 59 / terrestrial isopod gut symbiont (AY500250.1)	99%	87%
		29 (JN983628)	Uncultured bacterium clone zEL43 / Frasassi microbial mat community (DQ415836.1)	100%	95%
33 (JN983629)	Uncultured bacterium clone EDW07B005_144 / Edwards Aquifer microbial community (HM066626.1)	100%	96%		
34 (JN983630)	Uncultured Pseudomonadales bacterium clone LCM020 / bacterial colonization on adult black fly (JF733452.1)	100%	95%		
<i>N. frassasiensis</i>	Grotta Bella (GB), 2008	16 (JN983631)	Uncultured epsilon proteobacterium clone 4-138 / Alum Rock Park springs microbial community (GQ354915.1)	100%	99%
		20 (JN983632)	Uncultured bacterium clone VC105120 / Frasassi microbial mat community (JF747849.1)	100%	99%
		21 (JN983633)	Uncultured bacterium clone FC1_16S_47 / sulfidic karst systems microbial community (EU662454.1)	100%	99%
		27 (JN983634)	Uncultured Bacteroidetes bacterium clone UMAB-cl-134 / Antarctic Peninsula bacterial community (FR749759.1)	100%	95%
		11 (JN983635)	Uncultured bacterium clone EV818SWSAP25 / Kalahari Shield subsurface water bacterial community (DQ337068.1)	100%	94%
		3 (JN983636)	Uncultured bacterium clone VC1058 / Frasassi microbial mat community (JF747820.1)	100%	99%
		4 (JN983637)	Uncultured bacterium clone C3E / Chinese mitten crab (<i>Eriocheir sinensis</i>) intestinal bacteria (DQ856548.1)	99%	93%
		6 (JN983638)	Uncultured bacterium clone EV818SWSAP25 / Kalahari Shield subsurface water bacterial community (DQ337068.1)	100%	94%
8 (JN983639)	Uncultured Bacteroidetes bacterium clone UMAB-cl-134 / Antarctic Peninsula bacterial community (FR749759.1)	100%	95%		
<i>N. icitus</i>	Lago Claudia (LC), 2009	2 (JN983640)	Uncultured Bacteroidetes bacterium clone IC4044 / Arctic glacier ice bacterial community (HQ622740.1)	100%	96%
		5 (JN983641)	Unidentified proteobacterium clone B01-5 / deep-sea sediment bacterial community (AB015518.1)	100%	90%
		6 (JN983642)	Uncultured bacterium clone 8S7 / marine biofilm microbial community (JF272066.1)	100%	88%
		8 (JN983643)	Uncultured bacterium clone FL0428B_PF27 / sulfate-reducing cave biofilm microbial community (FJ716413.1)	100%	95%
<i>N. frassasiensis</i>	Pozzo dei Cristalli (PC), 2008	1 (JN983644)	Uncultured bacterium clone 2C228550 / aquatic bacterial community (EU800447.1)	100%	98%
		3 (JN983645)	Uncultured bacterium clone FrsF1199 / green phototrophic biofilm microbial community, Frasassi (JF747962.1)	100%	99%
		4 (JN983646)	Uncultured bacterium clone VC10574 / Frasassi microbial mat community (JF747856.1)	100%	99%
		5 (JN983647)	Uncultured bacterium clone lka79 / Frasassi microbial mat community (EF467575.1)	100%	99%
		6 (JN983648)	Uncultured bacterium clone 2C228550 / aquatic bacterial community (EU800447.1)	100%	98%
		7 (JN983649)	Uncultured bacterium clone 655952 / contaminated sediment microbial community (DQ404824.1)	100%	85%
		8 (JN983650)	Alpha proteobacterium A0904 16S / - (AF236001.1)	100%	93%
		49 (JN983651)	Uncultured bacterium clone 3BR-12AA / circumneutral freshwater microbial community (EU937911.1)	100%	96%
		51 (JN983652)	Uncultured bacterium clone SILK48 / Frasassi microbial mat community (EF467488.1)	100%	99%
		52 (JN983653)	Uncultured bacterium clone TP-SL-B-271 / Shule river permafrost bacterial community (HQ864209.1)	100%	96%
		53 (JN983654)	Uncultured bacterium clone BCWCWP1A42 / western Ghats (India) microbial community (FJ598380.1)	100%	96%
		56 (JN983655)	Uncultured bacterium clone WM4 / Frasassi mat microbial community (DQ133921.1)	100%	99%
		58 (JN983656)	Uncultured bacterium clone A156 / wastewater treatment plant microbial community (FJ660593.1)	100%	97%
		Ramo Sulfureo (RS), 2008	Ramo Sulfureo (RS), 2008	19 (JN983663)	Uncultured bacterium clone EV818SWSAP25 / Kalahari Shield subsurface water bacterial community (DQ337068.1)
25 (JN983664)	Uncultured rumen bacterium clone L102RT-2-D06 / bovine ruminal epithelial bacterial community (HQ400111.1)			100%	82%
33 (JN983665)	Uncultured bacterium clone CV104 / Frasassi acidic cave wall biofilm microbial community (EF530681.1)			100%	99%
Ramo Sulfureo (RS), 2009	Ramo Sulfureo (RS), 2009	11 (JN983657)	Uncultured bacterium clone FGL7S_B30 / Fayetteville Green Lake microbial community (FJ437988.1)	100%	98%
		14 (JN983658)	Uncultured bacterium clone A156 / wastewater treatment plant microbial community (FJ660593.1)	100%	98%
		16 (JN983659)	Uncultured bacterium clone A156 / wastewater treatment plant microbial community (FJ660593.1)	100%	97%
		17 (JN983660)	Uncultured bacterium clone Dianchi-34 / Dianchi lakeshore polluted water microbial community (HQ324880.1)	100%	95%
		26 (JN983661)	Uncultured epsilon proteobacterium clone / sulfur spring microbial community (AB425206.1)	100%	99%
		28 (JN983662)	Uncultured bacterium clone A156 / wastewater treatment plant microbial community (FJ660593.1)	100%	97%
Sorgente del Tunnel (ST), 2009	30 (JN983666)	Uncultured Bacteroidetes bacterium clone UMAB-cl-134 / Antarctic Peninsula bacterial community (FR749759.1)	100%	95%	

* Reference value for blastn hit ranking

Purple = Top nucleotide BLAST (blastn) hit matched by more than one sequence from the same clone library

Red = Top nucleotide BLAST (blastn) hit matched by several sequences from different clone libraries

Chapter 2:

Metabolic diversity among free-living and ectosymbiotic *Thiothrix* bacteria from the sulfidic Frasassi cave ecosystem

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2.1 Abstract

Niphargus is a genus of groundwater amphipods living in caves and springs throughout Europe. They are found in large numbers within the sulfide-rich Frasassi caves of Italy, which contain an ecosystem sustained by chemoautotrophy. Three *Niphargus* species live within Frasassi, all of which harbor filamentous sulfur-oxidizing *Thiothrix* ectosymbionts. The ectosymbionts belong to three distinct phylogenetic clades (T1–T3); T1 occurs on a single *Niphargus* species, whereas T2 and T3 are shared between different hosts. While free-living counterparts of T2 ectosymbionts were identified in cave microbial mats, the other two symbionts were detected exclusively on *Niphargus*.

The three *Niphargus* host species expose their ectosymbionts to various sulfide and oxygen regimes due to their different behaviors and microhabitat preferences. We postulated that T1–T3 would reveal different metabolic capabilities in their distinct ecological niches. Incubations with ^{13}C -labeled carbon substrates and ^{15}N -labeled nitrogen gas followed by Nano-scale Secondary Ion Mass Spectrometry (NanoSIMS) were used to examine the carbon and nitrogen metabolism of free-living and ectosymbiotic *Thiothrix*. The analyses revealed that T1 *Thiothrix* ectosymbionts are heterotrophs, whereas T2 and T3 are mixotrophs. In addition, T3 ectosymbionts are capable of nitrogen fixation. T2 and T3 ectosymbionts showed different carbon metabolisms when associated with different *Niphargus* host species. Moreover, in the presence of sulfide, autotrophic carbon fixation rates of T2 ectosymbionts were significantly greater than that of their free-living counterparts, suggesting an ecological advantage of the “hitchhiking” ectosymbiotic lifestyle.

2.2 Introduction

Ectosymbioses between sulfur-oxidizing bacteria and invertebrates are common in sulfidic marine environments and have evolved independently in several metazoan and protozoan phyla (Dubilier *et al.*, 2008). While free-living sulfur-oxidizing bacteria are restricted to narrow interfaces where sulfide and oxygen co-exist (Jørgensen and Revsbech, 1983), ectosymbionts are said to gain optimal growth conditions on the body surfaces of motile invertebrates due to being transported between sulfidic and

oxygenated environments (Polz *et al.*, 2000). Some authors have suggested that sulfur-oxidizing bacteria may even attain higher growth rates through intrinsic behaviors of their macroinvertebrate hosts. For example, the "dancing" behavior of the Yeti crab *Kiwa puravida*, which frequently waves its chelipeds in turbulent mixing waters at sulfidic hydrothermal vents, has been interpreted as active cultivation of ectosymbionts colonizing the crab's claws (Thurber *et al.*, 2011). Based on a modeling study, Roy and colleagues postulated that the peritrich ciliate *Zoothamnium niveum* induces a 100-fold increase in sulfide uptake rates of its ectosymbionts by generating water currents through its filter-feeding activity (Roy *et al.*, 2009). However, despite the building circumstantial evidence that sulfur-oxidizing ectosymbionts derive metabolic benefits when associating with motile hosts, experimental demonstration of this phenomenon is scarce.

Ectosymbioses between sulfur-oxidizing bacteria and gammaridean *Niphargus* amphipods have recently been reported from the freshwater Frasassi cave system in central Italy (Dattagupta *et al.*, 2009; Bauermeister *et al.*, 2012). The caves are forming by sulfuric acid-driven limestone dissolution and host an underground ecosystem sustained by chemoautotrophy (Sarbu *et al.*, 2000). Three *Niphargus* species, namely *Niphargus frasassianus*, *Niphargus ictus*, and *Niphargus montanarius*, inhabit the sulfidic cave waters (Flot *et al.*, 2010a; Karaman *et al.*, 2010), and all of them harbor filamentous *Thiothrix* bacteria on their exoskeletons (Bauermeister *et al.*, 2012).

Along with other sulfur-oxidizing epsilon- and gammaproteobacteria, *Thiothrix* is a dominant member of the microbial mat community in Frasassi (Macalady *et al.*, 2006). However, physicochemical cave water parameters determine ecological niches of various bacterial groups within the mats, and free-living *Thiothrix* bacteria form prominent populations only in turbulent streams with relatively high oxygen-to-sulfide ratios (Figure 2.1a; Macalady *et al.*, 2008). In contrast, *Thiothrix* ectosymbionts of the *Niphargus* amphipods thrive in the entire range of geochemical conditions and flow characteristics found in Frasassi cave waters (Dattagupta *et al.*, 2009; Bauermeister *et al.*, 2012). *N. frasassianus* lives in flowing streams, where it crawls among microbial mats and in adjacent oxygen-depleted sediments. *N. ictus* occurs in stagnant and deep lakes, where it periodically alternates between oxygenated top layers and sulfidic bottom waters. *N. montanarius* exclusively inhabits the shallow

waters of a small pool and is assumed to have only occasional access to sulfide (Figure 2.1b–d).

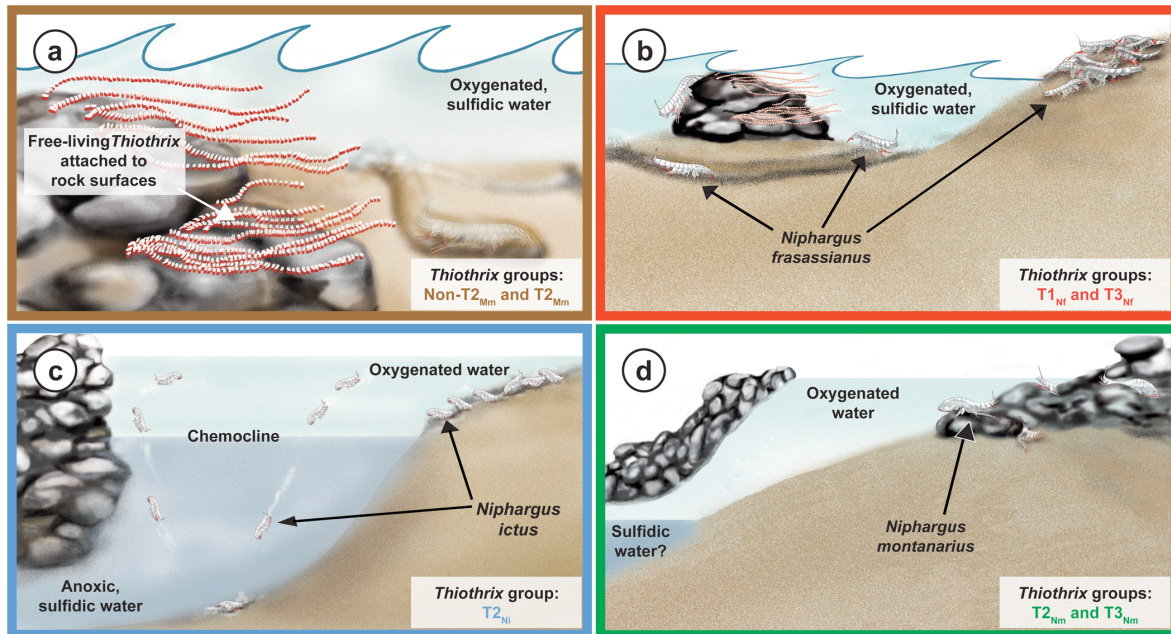


Figure 2.1. Environmental niche of free-living *Thiothrix* and habitat preferences and behaviors of *Niphargus* species in the Frasassi caves. (a) *Thiothrix* filaments attach to solid surfaces via basal holdfasts. The predominance of free-living *Thiothrix* populations in Frasassi is restricted to flowing waters with low sulfide-to-oxygen ratios. (b) *N. frassassianus* occurs in turbulent cave streams and occasionally shares its habitat with free-living *Thiothrix*. It predominantly crawls and hides in the hypoxic sediment, but can sometimes be found in damp sediment bordering the streams. (c) *N. ictus* inhabits stagnant water bodies, some of which have a distinct chemocline. It mostly swims or rests in the upper, oxygenated water zone. Occasionally, it dives down into deep, highly sulfidic water realms, remains on the hypoxic sediment surface for a few minutes, and comes up again. (d) *N. montanarius* is only found in Il Bugianardo, a small cave pool with oxygenated water, where it crawls in between gaps of boulders in the shallow-water zone. Sulfide in the water was detected on one out of seven trips to Il Bugianardo. *N. montanarius* is thus sporadically exposed to sulfidic conditions or might even have permanent access to a deep sulfidic water source. Artwork for this figure was done by Cornelia Hundertmark (Göttingen Center for Geosciences).

Thiothrix ectosymbionts of the three *Niphargus* species belong to three distinct phylogenetic clades (T1–T3), which are distributed among the hosts in a species-specific manner: clade T1 grows only on *N. frassassianus*, clade T2 is found on *N. ictus* and *N. montanarius*, and clade T3 can be present on all three *Niphargus* species (Figure 2.1; Bauermeister *et al.*, 2012). T1 and T3 ectosymbiont clades are distinct from free-living *Thiothrix* in Frasassi. However, *Thiothrix* filaments whose 16S rRNA gene sequence reveals only one consistent base-pair-mismatch with 16S sequences of ectosymbiont clade T2 have been detected in considerable amounts in cave microbial

mats (Bauermeister *et al.*, 2012). The co-occurrence of three host species with distinct habitat preferences and behaviors as well as closely related free-living and ectosymbiotic *Thiothrix* within one cave ecosystem provided us with the ideal opportunity to compare metabolic characteristics of *Thiothrix* within their different ecological niches.

Thiothrix are known to be metabolically versatile, and their capability of chemolithoautotrophic, heterotrophic, and mixotrophic growth has been demonstrated for various cultivated and uncultivated strains (Larkin and Shinabarger, 1983; Williams and Unz, 1989; Odintsova *et al.*, 1993; Tandoi *et al.*, 1994; Howart *et al.*, 1999; Nielsen *et al.*, 2000; Aruga *et al.*, 2002; Rossetti *et al.*, 2003; Chernousova *et al.*, 2009). We postulated that the different lifestyles of the *Thiothrix* bacteria (free-living and ectosymbiotic) as well as their association with differently behaving *Niphargus* hosts in Frasassi would be reflected in their metabolism. We used *in vivo* incubation experiments with isotopically labeled substrates followed by Nano-scale Secondary Ion Mass Spectrometry (NanoSIMS) to examine whether carbon and nitrogen incorporation capabilities vary between (I) free-living and ectosymbiotic *Thiothrix*, (II) different ectosymbiont clades present on the same *Niphargus* host species, and (III) members of the same ectosymbiont clade on different host species.

2.3 Materials and Methods

Sample collection

For an overview of the Frasassi caves and the collection sites, please refer to Bauermeister *et al.* (2012). *Niphargus* individuals were collected in July 2010 and March 2011 from three different cave locations within the Frasassi Grotta Grande del Vento-Grotta del Fiume complex; *N. frasassianus* from Sorgente del Tunnel, *N. ictus* from Lago Verde, and *N. montanarius* from Il Bugianardo. *Thiothrix* mat samples were obtained from cave locations Cave Spring, Grotta Bella, and Ramo Sulfureo in October 2011. All collection sites were accessed via technical spelunking routes. *Niphargus* were caught using small fishing nets and forceps as appropriate and transferred alive into polypropylene bottles filled with cave water from the respective collection site. Microbial mats were collected into falcon tubes using sterile pipettes. All samples

were kept at ambient cave temperatures (13–14 °C) during transfer to the laboratory in the nearby Osservatorio Geologico di Coldigioco field station, and experiments were conducted within 24 h of collection.

Isotope labeling experiments

Isotope labeling experiments were carried out in March and October 2011 using experimental conditions that had been optimized after preliminary tests conducted in July 2010 (Table 2.1). Either two conspecific *Niphargus* individuals or ~5 g of wet microbial mat were incubated in individual Wheaton® glass serum bottles (SIGMA-ALDRICH, Munich, Germany) containing carbon- and sulfide-free artificial Frasassi cave water (AFCW; 6.50 mM NaCl, 2.97 mM CaCl₂, 0.99 mM MgSO₄ · H₂O, 0.53 mM CaSO₄ · 2H₂O, 0.36 mM KCl, and 0.11 mM NH₄Cl; pH adjusted to 7.2). AFCW was supplemented with either sodium ¹²C-bicarbonate (NaH¹²CO₃; control experiment), sodium ¹³C-bicarbonate (NaH¹³CO₃; isotopic purity: 99 atom % ¹³C), or sodium ¹³C-lactate (¹³CH₃CH(OH)CO₂Na; isotopic purity: 99 atom % ¹³C). To every second bottle that contained a labeled carbon substrate, a concentrated sulfide stock solution (Na₂S · 9H₂O prepared in AFCW) was added.

Sulfide concentrations are very labile in water, as sulfide reacts with dissolved oxygen (Almgren and Hagström, 1974). Preliminary test series had been conducted in the lab to empirically determine (I) by which means and (II) how much volume of the sulfide stock solution had to be added to an incubation bottle to reach a final sulfide concentration similar to those measured in Frasassi cave waters (Macalady *et al.*, 2008; Flot *et al.*, 2010a). During the labeling experiments, a blank bottle containing no *Niphargus* animals or microbial mat sample was simultaneously prepared and, after addition of the sulfide stock solution, sampled for estimation of starting sulfide concentrations in all incubation waters. Dissolved sulfide concentration was measured with a DR2800 spectrophotometer (HACH LANGE, Düsseldorf, Germany) using the methylene blue method.

Incubation bottles were filled up to the top with AFCW and sealed without headspace using gas-tight butyl rubber stoppers (SIGMA-ALDRICH). Using an analytical syringe, 40 µL of ¹⁵N-nitrogen gas (isotopic purity: 98 atom % ¹⁵N) was

injected through the rubber stoppers and formed a small bubble in the incubation waters. All bottles were stored in a dark room at an ambient temperature of 16 °C. After 24 h of incubation, final sulfide concentrations in all incubation waters were measured immediately after removing the rubber stoppers. *Niphargus* individuals and microbial mats were washed in phosphate buffered saline (PBS), fixed for 3 h at 4 °C in 4% paraformaldehyde made in PBS, washed again three times in PBS, and finally stored in a 1:1 (v:v) PBS:ethanol solution.

N. montanarius individuals are found only in the Frasassi cave location Il Bugianardo (Bauermeister *et al.*, 2012) in small population sizes and are therefore extremely difficult to sample. During sample collection for March 2011 experiments, only one *N. montanarius* individual could be collected. Thus, in order to obtain a complete dataset, we used *N. montanarius* individuals incubated in ¹³C-bicarbonate and ¹³C-lactate without sulfide or ¹⁵N-nitrogen during the July 2010 trial experiments for NanoSIMS analyses (Table 2.1). 'Control individuals' of all three *Niphargus* species treated with ¹²C-bicarbonate were also used from the July 2010 incubations.

Table 2.1. Overview of the incubation experiments. Experiments in July 2010 were conducted without the addition of sodium sulfide or ¹⁵N-nitrogen gas to the incubation bottles. They served as trials and were optimized for the later experiments conducted in March and October 2011. However, due to the lack of *N. montanarius* individuals in March 2011 experiments, several samples from July 2010 incubations were ultimately used for NanoSIMS analyses. Tickmarks (✓) indicate the subsection of a sample to the respective treatment, and stars (*) mark samples finally analyzed with NanoSIMS. CS = cave location Cave Spring, GB = cave location Grotta Bella, RS = cave location Ramo Sulfureo.

		Experiments in July 2010			Experiments in March 2011			Experiments in October 2011		
Set-up	Incubation bottles	60 mL			40 mL			40 mL		
	Incubation water	25 mL			40 mL			40 mL		
	Agarose gel cubes	5 g			-			-		
	Head space	~30 mL			-			-		
	Sodium ¹² C-bicarbonate	6.6 mM			6.6 mM			6.6 mM		
	Sodium ¹³ C-bicarbonate	6.6 mM			6.6 mM			6.6 mM		
	Sodium ¹³ C-lactate	6.6 mM (L isomer)			6.6 mM (L/D isomer = 2:1)			6.6 mM (L/D isomer = 2:1)		
	Sodium sulfide	-			296 μM			282–477 μM		
	¹⁵ N-nitrogen gas	-			40 μL			40 μL (not applied to controls)		
Treatments	Specimen	<i>N. frasassianus</i>	<i>N. ictus</i>	<i>N. montanarius</i>	<i>N. frasassianus</i>	<i>N. ictus</i>	<i>N. montanarius</i>	Mat (CS)	Mat (GB)	Mat (RS)
	Amount per treatment	2	2	2	2	2	1	~5 g	~5 g	~5 g
	¹² C-bicarbonate (control)	✓*	✓*	✓*	-	-	-	✓	✓	✓*
	¹³ C-bicarbonate	✓	✓	✓*	✓*	✓*	-	✓	✓	✓*
	¹³ C-lactate	✓	✓	✓*	✓*	✓*	-	✓	✓	✓*
	¹³ C-bicarbonate + sulfide	-	-	-	✓*	✓*	✓*	✓	✓	✓*
	¹³ C-lactate + sulfide	-	-	-	✓*	✓*	-	✓	✓	✓*

Fluorescence *In Situ* Hybridization (FISH)

Several legs of each *Niphargus* individual were dissected under a stereomicroscope and ultrasonicated for 1 minute. Detached ectosymbionts as well as bacteria from microbial mat samples were filtered onto gold–palladium coated polycarbonate filters (GGTP type; 0.22 μm pore size; Millipore, Germany). 5mm diameter circles were excised from the center of each filter and subjected to fluorescence *in situ* hybridization (FISH) according to the standard protocol by Hugenholtz *et al.* (2002).

Oligonucleotide probe mixes T1Pmix, T2Pmix, and T3Pmix (Bauermeister *et al.*, 2012) were used to identify T1–T3 *Thiothrix* filaments obtained from *Niphargus* legs. All three probe mixes had previously shown optimal hybridization stringencies at 45% formamide concentration. Two probe mixes (differently labeled with fluorescein isothiocyanate and cyanine 3) could therefore be applied simultaneously to the same sample. This served to distinguish between T1 and T3 ectosymbionts of *N. frasassianus* (hereafter referred to as T1_{Nf} and T3_{Nf}) and between T2 and T3 ectosymbionts of *N. montanarius* (T2_{Nm} and T3_{Nm}). In accordance to previous results (Bauermeister *et al.*, 2012), *N. ictus* individuals from Lago Verde only harbored T2 *Thiothrix* filaments (T2_{Ni}). T2Pmix was further applied to microbial mat samples in combination with the general bacterial EUBmix probe (Daims *et al.*, 1999) to differentiate free-living T2 bacteria (T2_{Mm}) from other free-living *Thiothrix* (non-T2_{Mm}). T2_{Mm} filaments were abundant in mat samples from all three cave collection sites. NanoSIMS was performed only with samples from cave location Ramo Sulfureo in order to optimize analysis time.

Nano-scale Secondary Ion Mass Spectrometry (NanoSIMS)

Filter pieces were visually inspected on a laser micro-dissection microscope (Leica, Wetzlar, Germany). Using the laser, fluorescently labeled *Thiothrix* filaments of interest were marked with brackets and numbered. This allowed for their accurate retrieval during subsequent NanoSIMS analysis.

Samples were analyzed on a NanoSIMS 50L instrument (CAMECA SAS, Gennevilliers, France). Secondary ion images of $^{12}\text{C}^-$, $^{13}\text{C}^-$, $^{12}\text{C}^{14}\text{N}^-$, and $^{12}\text{C}^{15}\text{N}^-$ were recorded simultaneously for individual *Thiothrix* filaments using four electron

multipliers. Images were acquired as follows: First, a 50 x 50 μm image field was chosen to give an overview of the filament(s) to be analyzed. The sample was then sputtered with a 1.1–3.5 pA Cs^+ primary ion beam focused on a spot of ~ 120 nm diameter that was stepped over the sample in a 512 x 512 pixel raster with a counting time of 1 ms per pixel. The same region was rescanned 30 to 100 times, and the resulting planes were combined to create the final image.

Data processing was performed using the Look@NanoSIMS program (Polerecky *et al.*, 2012). In short, the different scanned planes of each ion mass were summed in floating 32 bits. NanoSIMS images were graphically displayed in a false-color scale ranging from black (intensity = 0) to red (maximum intensity per pixel adjusted so as to obtain good visual contrast). All scans of each image were realigned to correct for any drift of the sample stage during acquisition. Damaged planes were sorted out. Isotope ratio images ($^{13}\text{C}/^{12}\text{C}$, $^{12}\text{C}^{15}\text{N}/^{12}\text{C}^{14}\text{N}$) were created by adding the counts recorded for a chosen secondary ion or ion pair (^{13}C or $^{12}\text{C}^{15}\text{N}$) for each pixel over all scans and dividing the total counts by the total counts for a selected reference mass (^{12}C and $^{12}\text{C}^{14}\text{N}$, respectively). Individual cells within *Thiothrix* filaments were marked as regions of interest (ROIs) using the $^{12}\text{C}^{14}\text{N}$ images to define the cell outline. However, several *Thiothrix* filaments appeared to be encased by a sheath, which impeded the identification of individual cells. In these cases, ROIs of approximate cell size were marked within the filaments. For each ROI, the $^{13}\text{C}/^{12}\text{C}$ ratio and the $^{15}\text{N}/^{14}\text{N}$ ratio (inferred from the $^{12}\text{C}^{15}\text{N}/^{12}\text{C}^{14}\text{N}$) were calculated.

2.4 Results

All *Niphargus* individuals survived the 24h incubations. A total of 138 *Thiothrix* filaments divided into 1418 cells or ROIs were analyzed with NanoSIMS (Supplementary Table S2.1). Incorporation rates of ^{13}C -carbon and ^{15}N -nitrogen within the different *Thiothrix* groups were very heterogeneous. ^{13}C -carbon enrichments varied strongly not only between different *Thiothrix* filaments from the same group, but even among individual cells of the same filament (Figure 2.2).

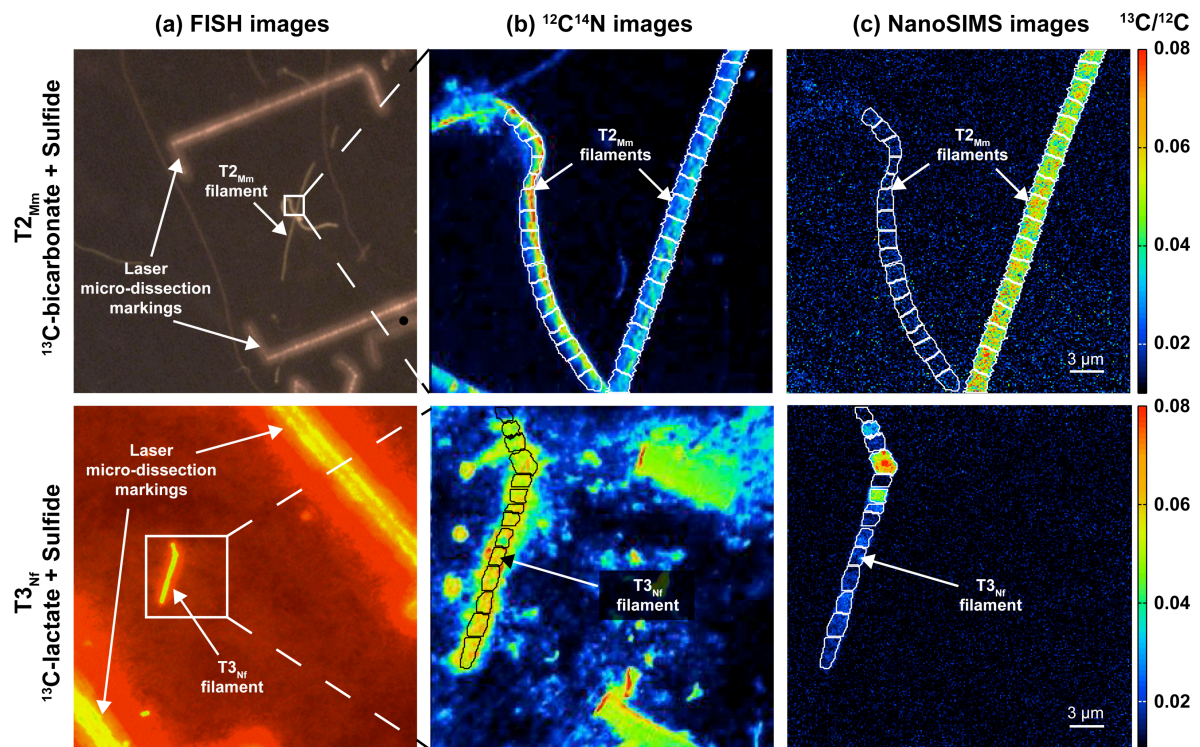


Figure 2.2. Heterogeneous carbon incorporation by *Thiothrix*. (a) FISH images of *Thiothrix* filaments marked by cyanine 3-labeled oligonucleotide probes. (b) $^{12}C^{14}N$ images of the same *Thiothrix* filaments. White and black lines delimit regions of interest (ROIs). (c) NanoSIMS images showing strongly varying $^{13}C/^{12}C$ ratios in different filaments of the same *Thiothrix* group (above) and in individual cells of the same *Thiothrix* filament (below).

^{13}C -bicarbonate incubations

$T2_{Mm}$ and $T2_{Ni}$ filaments displayed very high and similar ^{13}C enrichments after the 24h incubation with ^{13}C -bicarbonate (Figure 2.3a). Free-living non- $T2_{Mm}$ as well as ectosymbiotic $T3_{Nf}$ and $T3_{Nm}$ filaments showed lower, but still elevated $^{13}C/^{12}C$ ratios. No ^{13}C -bicarbonate incorporation was detected in $T1_{Nf}$ and $T2_{Nm}$ filaments.

When the incubation water was supplemented with sulfide (Figure 2.3b), $T2_{Mm}$ filaments incorporated substantially less ^{13}C -bicarbonate. Also, comparatively lower $^{13}C/^{12}C$ ratios were measured in non- $T2_{Mm}$ (Supplementary Table S2.1) and $T3_{Nf}$ filaments. ^{13}C enrichments in $T2_{Ni}$ and $T3_{Nm}$ filaments, in contrast, were substantially higher than those measured after incubation in non-sulfidic water. $T1_{Nf}$ and $T2_{Nm}$ filaments still revealed no uptake of inorganic carbon.

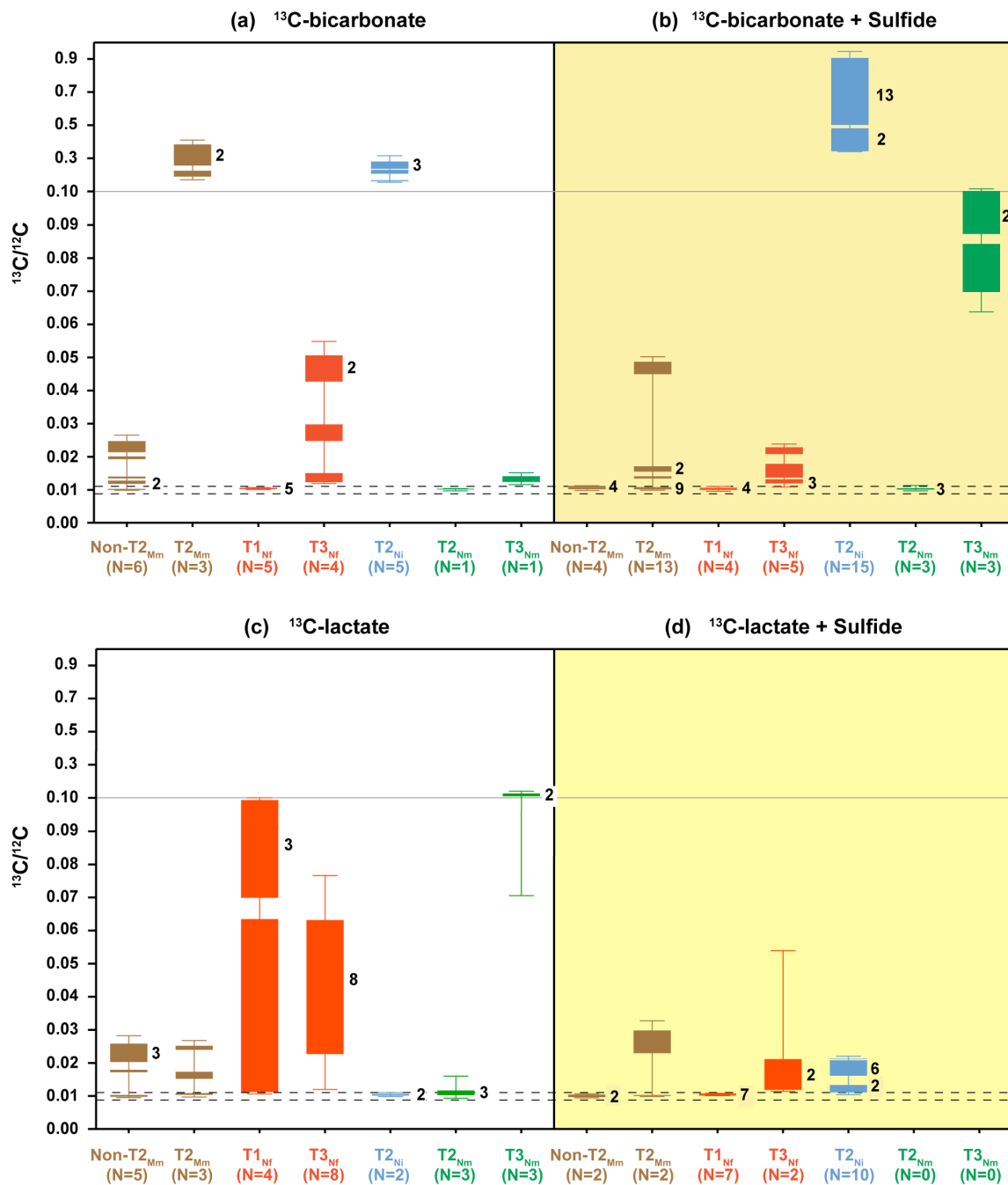


Figure 2.3. Box-and-whisker diagrams showing carbon isotopic ratios of the different *Thiothrix* groups after 24h incubations with ^{13}C -bicarbonate and ^{13}C -lactate. The range of $^{13}\text{C}/^{12}\text{C}$ ratios detected in control samples (treated with ^{12}C -bicarbonate) is indicated by the dashed lines. Yellow diagram backgrounds indicate *Thiothrix* incubations in the presence of sulfide. The gray horizontal line marks a change in scaling on the y-axis. Environments of the different *Thiothrix* groups are color-coded (brown = microbial mat, red = *N. frassianus*, blue = *N. ictus*, green = *N. montanarius*). N = Number of analyzed filaments. Bottom and top of each box in the diagrams delimit the range from the 25th to the 75th percentile of $^{13}\text{C}/^{12}\text{C}$ ratios measured within a single *Thiothrix* filament. Numbers next to certain boxes indicate an overlap of data from several filaments; in these cases, bottom and top of the boxes represent the lowest 25th and the highest 75th percentile measured among all contributing filaments. The ends of the whiskers (= straight lines connecting the boxes) mark the minimum and the maximum $^{13}\text{C}/^{12}\text{C}$ ratios measured.

¹³C-lactate incubations

In the absence of sulfide, organic carbon from ¹³C-lactate was incorporated by all *Thiothrix* groups except for T2_{Ni} (Figure 2.3c). Particularly high ¹³C/¹²C ratios were measured in cells of T1_{Nf}, T3_{Nf}, and T3_{Nm} filaments. Few cells in only one out of three analyzed T2_{Nm} filaments revealed slight ¹³C enrichments (Supplementary Table S2.1).

When sulfide was present in the incubation water (Figure 2.3d), no ¹³C-lactate incorporation was detected in non-T2_{Mm} and T1_{Nf} filaments. ¹³C/¹²C ratios of T3_{Nf} were in the lower range of ratios measured for the same group with ¹³C-lactate alone. No apparent change of organic carbon incorporation rates was found in T2_{Mm} filaments. In contrast to the non-sulfidic treatment, ¹³C-lactate incorporation was now also detected in T2_{Ni} filaments. Due to the scarcity of *N. montanarius* individuals, data for lactate incorporation by T2_{Nm} and T3_{Nm} filaments in the presence of sulfide are missing in our study.

¹⁵N-nitrogen incubations

Ectosymbiotic T3_{Nf} and T3_{Nm} filaments revealed increased ¹⁵N/¹⁴N ratios after incubation with ¹⁵N-nitrogen gas (Figure 2.4). Highest ratios were measured in two T3_{Nf} filaments after incubation with ¹³C-bicarbonate in the absence of sulfide. Individual ROIs of these filaments revealed ¹⁵N enrichment of approximately 240% relative to control values (Supplementary Table S2.1). Due to the scarcity of *N. montanarius* animals, ¹⁵N/¹⁴N data for T3_{Nm} filaments could be obtained only from ¹³C-bicarbonate + sulfide incubations. Filaments of other *Thiothrix* groups did not show ¹⁵N incorporations (Supplementary Table S2.1).

2.5 Discussion

This study revealed considerable diversity in metabolic characteristics of *Thiothrix* bacteria occupying different niches within the Frasassi cave ecosystem. The high variability of ¹³C/¹²C and ¹⁵N/¹⁴N ratios observed among different filaments from the same *Thiothrix* treatment group and among various cells within the same filaments (Figures 2.2–2.4) was similar to findings reported from other filamentous microbial species (Musat *et al.*, 2008) and may have been caused by different metabolic states of

the *Thiothrix* cells. Due to these strong heterogeneities, we interpreted the results qualitatively instead of conducting statistical analyses to compare mean incorporation rates of different *Thiothrix* groups.

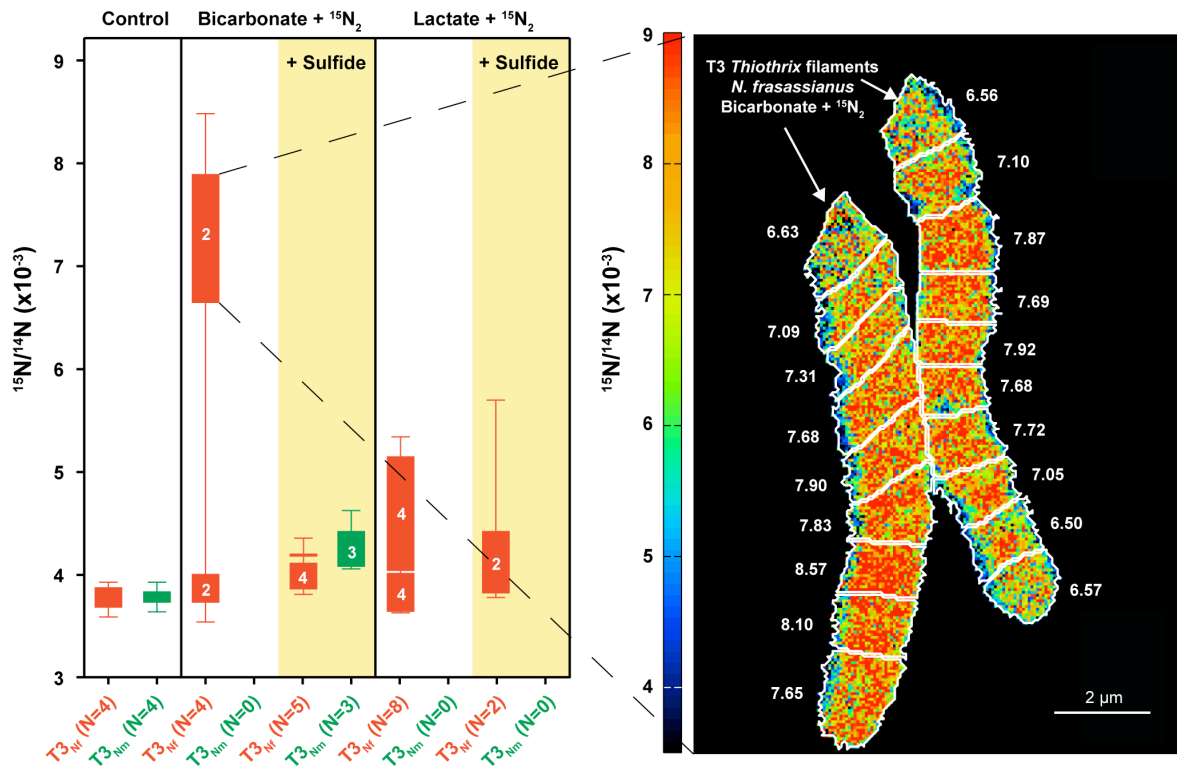


Figure 2.4. Nitrogen fixation by T3 *Thiothrix* ectosymbionts of *N. frassassianus* and *N. montanarius*. Left: Box-and-whisker diagram showing nitrogen isotopic ratios of T3 *Thiothrix* filaments after 24h incubations with ¹⁵N₂. 'Control' filaments were incubated with ¹²C-bicarbonate and no ¹⁵N₂ gas. Yellow diagram backgrounds indicate incubations in the presence of sulfide. The two different *Niphargus* host species of T3 are color-coded (red = *N. frassassianus*, green = *N. montanarius*). N = Number of analyzed filaments. Bottom and top of each box in the diagram delimit the range from the 25th to the 75th percentile of ¹⁵N/¹⁴N ratios measured within a single *Thiothrix* filament. Numbers within most of the boxes indicate an overlap of data from several filaments; in these cases, bottom and top of the boxes represent the lowest 25th and the highest 75th percentile measured among all contributing filaments. The ends of the whiskers (= straight lines connecting the boxes) mark the minimum and the maximum ¹⁵N/¹⁴N ratios measured. Right: NanoSIMS image of two T3 *Thiothrix* filaments from *N. frassassianus* (T3_{Nf}) after 24h incubation with bicarbonate and ¹⁵N₂. White lines delimit regions of interest (ROIs) as defined during NanoSIMS data evaluation, and values next to the ROIs indicate average ¹⁵N/¹⁴N ratios.

T2 ectosymbionts of *N. ictus* (T2_{Ni}) have previously been shown to be capable of chemoautotrophy (Dattagupta *et al.*, 2009). In this study, most free-living as well as ectosymbiotic *Thiothrix* groups analyzed were found to incorporate ¹³C from both bicarbonate and lactate, and thus to be capable of mixotrophy (Figure 2.3;

Supplementary Table S2.1). Exceptions were T1 ectosymbionts of *N. frasassianus* (T1_{Nf}) and T2 ectosymbionts of *N. montanarius* (T2_{Nm}), which were found to be obligately heterotrophic.

Nitrogen fixation has been previously reported in *Thiothrix caldifontis* cultures using the acetylene reduction assay (Chernousova *et al.*, 2009). In our study, we could directly demonstrate the incorporation of molecular nitrogen into T3 *Thiothrix* cells. In addition to being mixotrophic, T3 *Thiothrix* ectosymbionts of both *N. frasassianus* and *N. montanarius* (T3_{Nf} and T3_{Nm}) are capable of nitrogen fixation (Figure 2.4). This metabolic versatility of T3 ectosymbionts may be the reason why they can colonize all three Frasassi-dwelling *Niphargus* species, which expose them to very different geochemical environments (Figure 2.1; Bauermeister *et al.*, 2012). Consistent with this, the least versatile, obligately heterotrophic T1 ectosymbionts are found on only one host, *N. frasassianus*.

Lactate was chosen as the organic carbon substrate in this study because it is utilized by most cultivated *Thiothrix* strains (Williams and Unz, 1989; Odintsova *et al.*, 1993; Rossetti *et al.*, 2003; Chernousova *et al.*, 2009). ¹³C-lactate enrichments in T1 and T3 filaments from *N. frasassianus* (T1_{Nf} and T3_{Nf}) were among the highest in the *Thiothrix* groups tested here. In its natural habitat, *N. frasassianus* crawls in hypoxic, sulfidic microbial mats and occasionally lies on the bordering damp sediment while rapidly moving its swimming legs, presumably to hyperventilate (Figure 2.1b). Two different *Niphargus* species, *N. rhenorhodanensis* and *N. virei*, have been shown to respond to severe hypoxia with excretion of lactate across their exoskeletons (Hervant *et al.*, 1995, 1996). It is possible that *N. frasassianus* also secretes lactate and that its ectosymbionts are particularly adapted to utilize this substrate.

T2 *Thiothrix* are found in three different niches in the Frasassi cave ecosystem: as free-living bacteria in microbial mats attached to limestone boulders and as ectosymbionts of *N. ictus* and *N. montanarius* (Figure 2.1). Our data indicate striking differences in the metabolism of T2 *Thiothrix* in the three niches. T2 on *N. montanarius* (T2_{Nm}) appear to be obligate heterotrophs with very low organic carbon incorporation rates (Figure 2.3c). In contrast, T2 filaments in mats (T2_{Mm}) and on *N. ictus* (T2_{Ni}) showed high and similar rates of autotrophic carbon fixation in the absence of supplementary sulfide (Figure 2.3a). Previous investigations of *Thiothrix*

filaments from Frasassi microbial mats and from *N. ictus* revealed abundant sulfur globules in their cells (Macalady *et al.*, 2006; Dattagupta *et al.*, 2009). It is likely that T2_{Mm} and T2_{Ni}, like other *Thiothrix*, can oxidize these elemental sulfur reserves to sulfate to support chemoautotrophy (Larkin and Strohl, 1983).

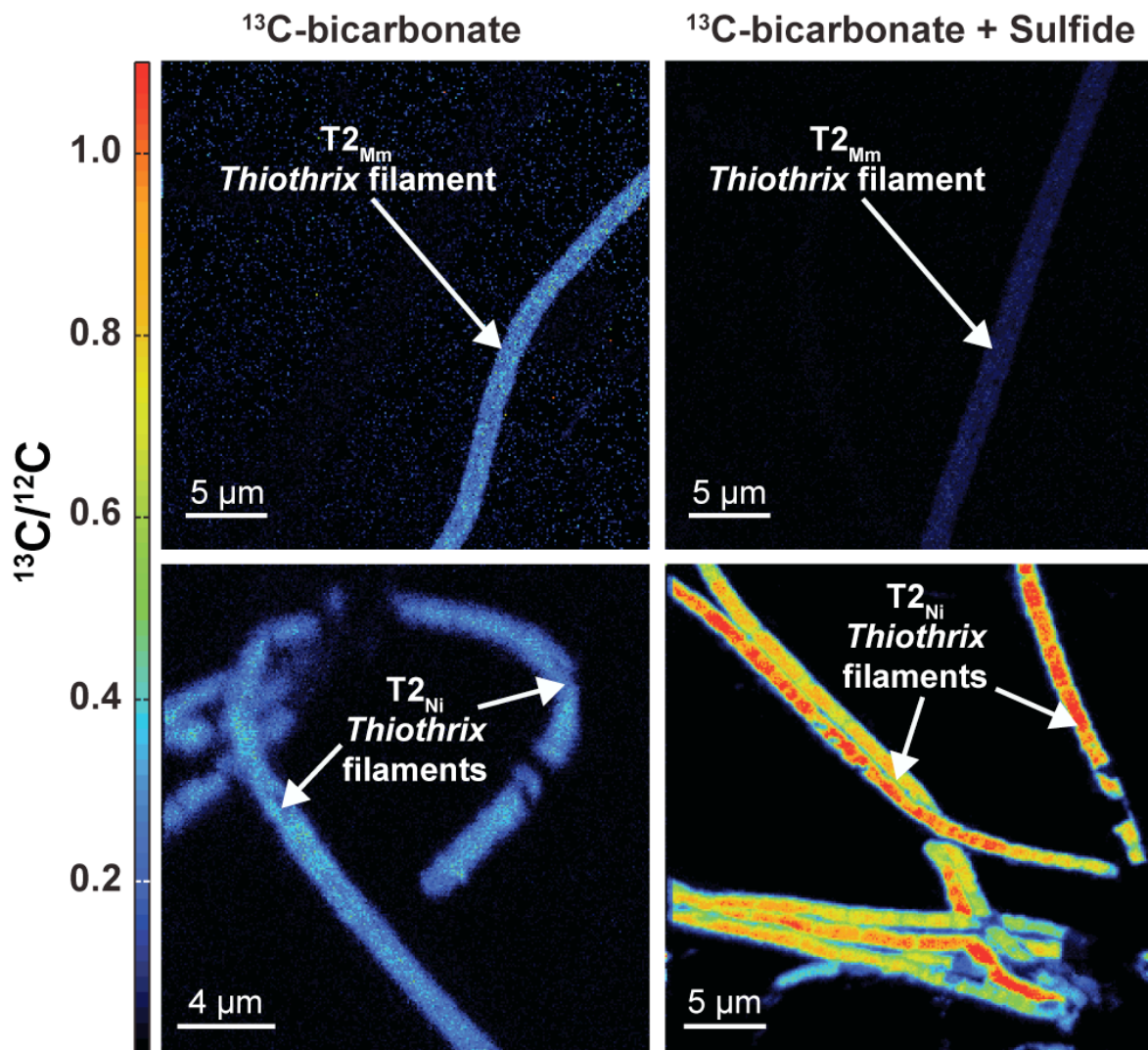


Figure 2.5. NanoSIMS images of $^{13}\text{C}/^{12}\text{C}$ ratios in free-living T2_{Mm} and ectosymbiotic T2_{Ni} *Thiothrix* filaments after 24h incubation with ^{13}C -bicarbonate. The presence of sulfide in the incubation water caused a decrease in ^{13}C uptake by T2_{Mm} (top right), but an increase in ^{13}C incorporation by T2_{Ni} filaments (bottom right).

The addition of sulfide to the incubation bottles resulted in opposite effects on carbon fixation rates of T2_{Mm} and T2_{Ni} (Figures 2.3b and 2.5). While sulfide addition caused a 8–199% increase in inorganic carbon fixation rates of T2_{Ni}, it largely

impeded chemoautotrophy by T2_{Mm}. *Thiothrix* ectosymbionts of *N. ictus* thrive in stagnant waters containing up to 415 μM sulfide (Flot *et al.*, 2010a), whereas *Thiothrix*-dominated microbial mats are restricted to turbulent streams and rarely occur at sulfide concentrations beyond 210 μM (Macalady *et al.*, 2008). Consistent with this, our results showed that exposure to 389–477 μM sulfide in the incubation bottles (Supplementary Table S2.1) severely diminished autotrophic carbon fixation by T2_{Mm}. *Thiothrix* mat samples sank to the bottom when introduced to the experimental bottles and possibly experienced oxygen deprivation or sulfide toxicity when incubated with sulfide. In contrast, *N. ictus* individuals swam around in the water during the incubation period. Gammaridean amphipods can enhance oxygen circulation around their gills through water currents produced by their legs (Dahl, 1977; Trontelj *et al.*, 2012). T2_{Ni} filaments are predominantly attached to the legs and antennae of *N. ictus* (Dattagupta *et al.*, 2009; Bauermeister *et al.*, 2012). It is possible that the movements of their hosts during swimming provided sufficient oxygen in the ectosymbionts' microenvironment to support high sulfide oxidation and autotrophic carbon fixation rates when incubated with sulfide. Thus, in their natural environment, T2 *Thiothrix* may derive a distinct metabolic advantage from “hitchhiking” on *N. ictus*, as they can apparently maintain high rates of chemoautotrophy even when their host swims into sulfidic zones of Frasassi cave lakes (Figure 2.1c).

T2_{Ni} and T2_{Nm} are indistinguishable at the 16S rRNA gene sequence level, and the 16S sequences of T2_{Mm} have only one consistent base difference with the ectosymbiont sequences (Bauermeister *et al.*, 2012). Our study indicates that different *Thiothrix* ecotypes with almost identical 16S rRNA gene sequences can have highly disparate physiologies or metabolic rates. This is similar to what has previously been shown for marine *Prochlorococcus* cyanobacteria (Moore *et al.*, 1998; Johnson *et al.*, 2006) as well as for the freshwater bacterium *Brevundimonas alba* (Jaspers and Overmann, 2004).

Moreover, autotrophic carbon fixation rates of free-living T2 *Thiothrix* (T2_{Mm}) were substantially higher than those of other *Thiothrix* bacteria (non-T2_{Mm}) in the same mat samples (Figure 2.3a). T2 ectosymbionts of *N. ictus* (T2_{Ni}) likely evolved from free-living ancestors in Frasassi cave microbial mats (Bauermeister *et al.*, 2012). One speculation is that the high autotrophic growth rate of T2 may have given it a selective

advantage over other microbial mat *Thiothrix* during establishment of the ectosymbiosis with *N. ictus*.

Another important observation is that different *Thiothrix* ectosymbionts occupying the same *Niphargus* host had distinct metabolic characteristics. For example, T1_{Nf} and T3_{Nf} co-exist on *N. frasassianus*; while T1_{Nf} is obligately heterotrophic, T3_{Nf} is a mixotroph capable of nitrogen fixation. Similarly, T2_{Nm} and T3_{Nm} co-exist on *N. montanarius*, with T3_{Nm} showing substantially higher rates of bicarbonate and lactate incorporation than T2_{Nm} (Figure 2.3). The Frasassi cave ecosystem is highly dynamic with temporally and spatially changing physicochemical parameters (Macalady *et al.*, 2008). Perhaps the metabolically disparate ectosymbionts can co-exist on their *Niphargus* hosts because different environmental conditions are favorable for the distinct clades. Maintenance of multiple ectosymbionts with varying metabolic capabilities would also be beneficial for *Niphargus* in case nutritional exchange occurs between the symbionts and the host. Direct transfer of soluble organic compounds of ectosymbiotic origin across the host integument was recently demonstrated for the hydrothermal vent shrimp *Rimicaris exoculata* (Ponsard *et al.*, 2012). It remains to be investigated whether a similar process occurs in the *Niphargus-Thiothrix* ectosymbioses.

2.6 Acknowledgements

The authors thank Alessandro Montanari for logistical support during fieldwork. We are grateful to Mahesh S. Desai and Pia Sternisek for assistance in conducting the isotope labeling experiments. Sincere thanks are also addressed to Tomas Vagner for his help with the NanoSIMS analyses, and to Rachel Foster, Mahesh S. Desai, and Jean-François Flot for helpful discussions. Cornelia Hundertmark created the drawings of the *Thiothrix* ecological niches. Field and lab work of this study were funded by the Excellence Initiative of the German Research Foundation (DFG). NanoSIMS analyses were conducted at the MPI Bremen, Germany, and financially supported by the Max Planck Society.

2.7 Supplementary Material

Supplementary Table S2.1. Tabular overview of measured data from incubation experiments and subsequent NanoSIMS analyses.

Thiothrix source	Carbon source	Nitrogen	Sulfide ¹ [μM S ²⁻]	Thiothrix group targeted ²	# of ¹³ C-enriched		Mean ¹³ C/ ¹² C in all ROIs ³	# of ¹⁵ N-enriched		Mean ¹⁵ N/ ¹⁴ N in all ROIs ³
					filaments	ROIs ³		filaments	ROIs ³	
Microbial mat	¹² C-bicarbonate	n.a.	n.a.	non-T2 _{Mm}	0/1	0/16	0.01020±0.00018	0/1	0/16	0.00363±0.00008
				T2 _{Mm}	0/2	0/33	0.00973±0.00033	0/2	0/33	0.00354±0.00019
	¹³ C-bicarbonate	¹⁵ N ₂	n.a.	non-T2 _{Mm}	5/6	55/62	0.01493±0.00466	0/6	0/62	0.00362±0.00017
				T2 _{Mm}	3/3	44/44	0.27797±0.06921	0/3	0/44	0.00344±0.00018
				477–389	non-T2 _{Mm}	4/4	18/52	0.01075±0.00033	0/4	0/52
				T2 _{Mm}	7/13	88/191	0.01562±0.01013	0/13	0/191	0.00366±0.00013
	¹³ C-lactate	¹⁵ N ₂	n.a.	non-T2 _{Mm}	4/5	54/60	0.02109±0.00460	0/5	0/60	0.00367±0.00009
				T2 _{Mm}	3/3	36/50	0.01736±0.00601	0/3	0/50	0.00359±0.00032
				282–178	non-T2 _{Mm}	0/2	0/21	0.01015±0.00035	0/2	0/21
			T2 _{Mm}	1/2	11/26	0.01711±0.00852	0/2	0/26	0.00364±0.00012	
<i>Niphargus frassianus</i>	¹² C-bicarbonate	n.a.	n.a.	T1 _{Nr}	0/1	0/5	0.01042±0.00012	0/1	0/5	0.00357±0.00009
				T3 _{Nr}	0/4	0/24	0.01037±0.00024	0/4	0/24	0.00378±0.00008
	¹³ C-bicarbonate	¹⁵ N ₂	n.a.	T1 _{Nr}	0/5	0/49	0.01043±0.00014	0/5	0/49	0.00372±0.00009
				T3 _{Nr}	4/4	38/38	0.03432±0.01435	4/4	26/38	0.00566±0.00181
				296–204	T1 _{Nr}	0/4	0/48	0.01033±0.00032	0/4	0/48
				T3 _{Nr}	5/5	22/23	0.01434±0.00335	5/5	17/23	0.00402±0.00014
	¹³ C-lactate	¹⁵ N ₂	n.a.	T1 _{Nr}	4/4	58/63	0.06617±0.02618	0/4	0/63	0.00383±0.00004
				T3 _{Nr}	8/8	67/67	0.04188±0.01486	7/8	38/67	0.00418±0.00044
				296–202	T1 _{Nr}	0/7	0/49	0.01044±0.00018	0/7	0/49
			T3 _{Nr}	2/2	21/21	0.01993±0.01017	2/2	17/21	0.00431±0.00041	
<i>Niphargus ictus</i>	¹² C-bicarbonate	n.a.	n.a.	T2 _{Nr}	0/2	0/18	0.01046±0.00021	0/2	0/18	0.00372±0.00018
	¹³ C-bicarbonate	¹⁵ N ₂	-	T2 _{Nr}	5/5	42/42	0.24492±0.03357	0/5	0/42	0.00359±0.00011
			296–194	T2 _{Nr}	15/15	99/99	0.64414±0.14277	0/15	0/99	0.00317±0.00021
	¹³ C-lactate	¹⁵ N ₂	-	T2 _{Nr}	0/2	0/22	0.01038±0.00030	0/2	0/22	0.00370±0.00012
296–197			T2 _{Nr}	10/10	66/69	0.01694±0.00341	0/10	0/69	0.00374±0.00010	
<i>Niphargus montanarius</i>	¹² C-bicarbonate	n.a.	n.a.	T2 _{Nm}	0/1	0/15	0.01021±0.00011	0/1	0/15	0.00370±0.00005
				T3 _{Nm}	0/4	0/45	0.01058±0.00019	0/4	0/45	0.00379±0.00006
	¹³ C-bicarbonate	n.a.	n.a.	T2 _{Nm}	0/1	0/5	0.01016±0.00025	0/1	0/5	0.00351±0.00022
				T3 _{Nm}	1/1	9/9	0.01339±0.00122	0/1	0/9	0.00373±0.00012
				296–220	T2 _{Nm}	0/3	0/43	0.01029±0.00028	0/3	0/43
	T3 _{Nm}	3/3	35/35		0.09148±0.01151	3/3	35/35	0.00425±0.00015		
	¹³ C-lactate	n.a.	n.a.	T2 _{Nm}	1/3	7/40	0.01086±0.00113	0/3	0/40	0.00374±0.00012
T3 _{Nm}				3/3	34/34	0.12099±0.01236	0/3	0/34	0.00376±0.00010	

¹ Sulfide concentration ranges refer to values measured before and after the 24h incubations

² 'non-T2_{Mm}' are *Thiothrix* filaments in microbial mats that did not hybridize to the T2-specific FISH probe NSPT2

³ ROIs = Regions Of Interest

Yellow-grounded cells indicate data from incubations in sulfidic water

Chapter 3:

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

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3.1 Abstract

Sulfide is toxic to most aerobic organisms. Two species of the groundwater amphipod genus *Niphargus* are found in high densities in the sulfide-rich waters of the Frasassi caves in central Italy. Both species harbor sulfide-oxidizing *Thiothrix* ectosymbionts predominantly attached to their pereopods. In this study, we examined whether the *Niphargus* hosts depend on their *Thiothrix* ectosymbionts for sulfide detoxification. Sulfide toxicity was compared between *Niphargus* with ectosymbionts and those whose symbionts had been killed using antibiotic treatment. We found that the ectosymbionts have no impact on the sulfide tolerance of their hosts, implying that the amphipods have an innate ability to tolerate sulfide. Both tested *Niphargus* species had exceptionally high sulfide tolerances compared to other amphipod species studied so far.

3.2 Introduction

Sulfide is toxic to most aerobic organisms, as it binds to cytochrome *c* oxidase, thereby inhibiting mitochondrial electron transport (Nicholls, 1975). It also impedes oxygen transport in many organisms by blocking binding sites on hemoglobin (Evans, 1967). A variety of invertebrates living in sulfide-rich marine environments employ effective strategies to avoid sulfide poisoning, some of which involve microbial symbionts. For example, hydrothermal vent tubeworms and shallow-water clams host intracellular endosymbionts that oxidize hydrogen sulfide to non-toxic sulfur compounds (Anderson *et al.*, 1987; Wilmot and Vetter, 1990). A sulfide-detoxifying role has further been suggested, but not experimentally verified, for ectosymbiotic bacteria, like those covering the gill surfaces of hydrothermal vent shrimp (Tokuda *et al.*, 2008).

The first non-marine ectosymbiosis between an aquatic invertebrate and sulfur-oxidizing bacteria was reported from the subterranean Frasassi cave system in central Italy (Dattagupta *et al.*, 2009). Two groundwater amphipod species of the genus *Niphargus*, namely *Niphargus ictus* Karaman 1985 and *Niphargus frasassianus* Karaman, Borowsky, Dattagupta 2010, inhabit the sulfidic waters of the Frasassi caves (Flot *et al.*, 2010a), and both of them harbor filamentous sulfur-oxidizing *Thiothrix*

bacteria on their chitinous exoskeletons (Bauermeister *et al.*, 2012). Sulfide concentrations in Frasassi cave waters range from 0.1 to 0.5 mM (Galdenzi *et al.*, 2008), which is far higher than the critical level that inhibits mitochondrial respiration of most aquatic organisms (Bagarinao, 1992). As high densities of sulfide-oxidizing *Thiothrix* filaments are attached to the *Niphargus* pereopods, it has been suggested that they may prevent sulfide diffusion across the amphipod gills (Dattagupta *et al.* 2009). The aim of the present study was to examine if *N. ictus* and *N. frasassianus* depend on sulfide oxidation by their *Thiothrix* ectosymbionts for survival in the sulfidic Frasassi cave waters. Individuals of the two *Niphargus* species were exposed to cave water with gradually increasing sulfide concentrations, and the sulfide tolerance of *Thiothrix*-hosting *Niphargus* was compared to that of individuals whose ectosymbionts had previously been killed by antibiotic treatment.

3.3 Materials and 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 Sorgente del Tunnel, respectively (for a map of the Frasassi caves, see Bauermeister *et al.*, 2012). Animals were caught using small fishing nets and forceps, as appropriate, and transferred into 500mL polypropylene bottles filled with cave water from the respective sampling site. Additionally, non-sulfidic cave water was collected from the cave pool Il Bugianardo (BG). *Niphargus* individuals and cave water samples were kept at ambient cave temperature during transfer to the laboratory.

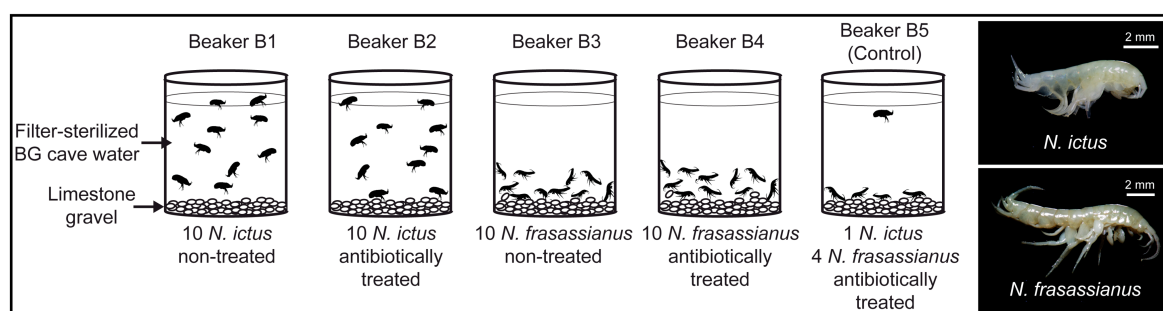
The experiment was conducted within 24 h of collection at the Osservatorio Geologico di Coldigioco field station, in a room with an ambient air temperature of ~16 °C. Three of the 24 *N. ictus* individuals died during transfer, so we could include only one instead of four *N. ictus* control individuals (details below). Five plastic 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).

An antibiotic solution (12.5 mg/L) was prepared from streptomycin sulfate (Roth, Karlsruhe, Germany) dissolved in filter-sterilized BG cave water. 11 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 and Unz, 1985). After the antibiotic treatment, *Niphargus* individuals were briefly bathed in filter-sterilized BG cave water to wash off any excess streptomycin before transfer to the experimental beakers.

A sulfide stock solution (SSS) was prepared from 60 mg of sodium sulfide nonahydrate ($\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$; SIGMA-ALDRICH, Steinheim, Germany) dissolved in 45 mL of filter-sterilized BG cave water. Antibiotically treated and non-treated *Niphargus* were divided into B1–B5 as shown in Figure 3.1. Antibiotically treated *N. ictus* (N=1) and *N. frasassianus* (N=5) individuals in B5 served as control animals to check whether the streptomycin treatment caused mortality in the absence of sulfide addition. Starting one hour after introducing the *Niphargus* individuals, a sterile pipette was used to periodically inject several milliliters of SSS to B1–B4. 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 and Morris, 1972). The same volume of filter-sterilized, non-sulfidic BG cave water was added similarly to B5, serving as control treatment. Prior to each addition of SSS, the alive *Niphargus* in each beaker were counted. Dissolved oxygen, pH-value, conductivity, and temperature in the waters were measured 15 minutes after each SSS injection using HQ40d multimeter sensors (HACH LANGE, Düsseldorf, Germany). Dissolved sulfide concentrations were measured at the same time using the methylene blue method (HACH LANGE).

Figure 3.1. Experimental set-up of this study.



The experiment was conducted over a total time span of 22 hours. By periodical injection of SSS, sulfide concentrations in the incubation waters of B1–B4 were raised 12 times within the first 14 h. After the third injection of SSS, increasing pH-values of the incubation waters were repeatedly adjusted to ~8 by addition of a few drops of concentrated hydrochloric acid (Supplementary Table S3.1). *Niphargus* individuals were observed continuously throughout the experiment to detect mortality. Animals were concluded to be dead if they remained immobile despite being nudged with a pipette tip.

After completion of the experiment, several antibiotically treated and non-treated *Niphargus* individuals were prepared for being examined with scanning electron microscopy (SEM). They were transferred into individual eppendorf tubes filled with 2.5% glutaraldehyde solution (SIGMA-ALDRICH) made in filter-sterilized BG cave water. Samples were sequentially dehydrated in ethanol concentrations from 30% to 90%, with a final dehydration in hexamethyldisilazane (SIGMA-ALDRICH) for 5–10 minutes. They 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).

3.4 Results

Scanning electron microscopy (SEM) showed that non-treated *Niphargus* individuals harbored numerous intact *Thiothrix* filaments on their body, whereas animals treated with streptomycin featured empty filament sheaths or remnants of *Thiothrix* holdfasts still attached to their exoskeletons (Figure 3.2). These observations confirmed the effective killing of *Thiothrix* ectosymbionts by the antibiotic treatment.

All antibiotically treated as well as non-treated animals survived until 9:30 h after the start of the experiment. At that point of time, one antibiotically treated *N. ictus* individual in B2 was found to be dead at a sulfide concentration of ~4 mM and an oxygen content of ~29 μM (Figure 3.3; Supplementary Table S3.1). Also, many of the remaining *Niphargus* animals in B1–B4 appeared torpid. Most *N. frasassianus* individuals were found on the bottoms of B3 and B4 and to be barely moving, whereas

N. ictus individuals in B1 and B2 mainly resided near the water surface, lying on their backs and fanning swiftly with their pleopods.

After the eighth addition of SSS, the water in B1–B4 had turned turbid, and a second *N. ictus* individual from B2 had died. Many of the other *Niphargus* were motionless, but still reacted slightly upon nudging. When the pH-values of the incubation waters were adjusted to ~8, the whitish precipitate disappeared, and few minutes later, all alive *Niphargus* individuals became active again.

After 13:45 h, a third antibiotically treated *N. ictus* individual in B2 and four antibiotically treated *N. frassianus* individuals in B4 had died. At that time, dissolved sulfide in the incubation waters of B2 and B4 had reached concentrations of almost 11.5 mM, and oxygen concentrations were 14 μM and 12 μM , respectively. All non-treated *N. ictus* and *N. frassianus* individuals in B1 and B3 were still alive.

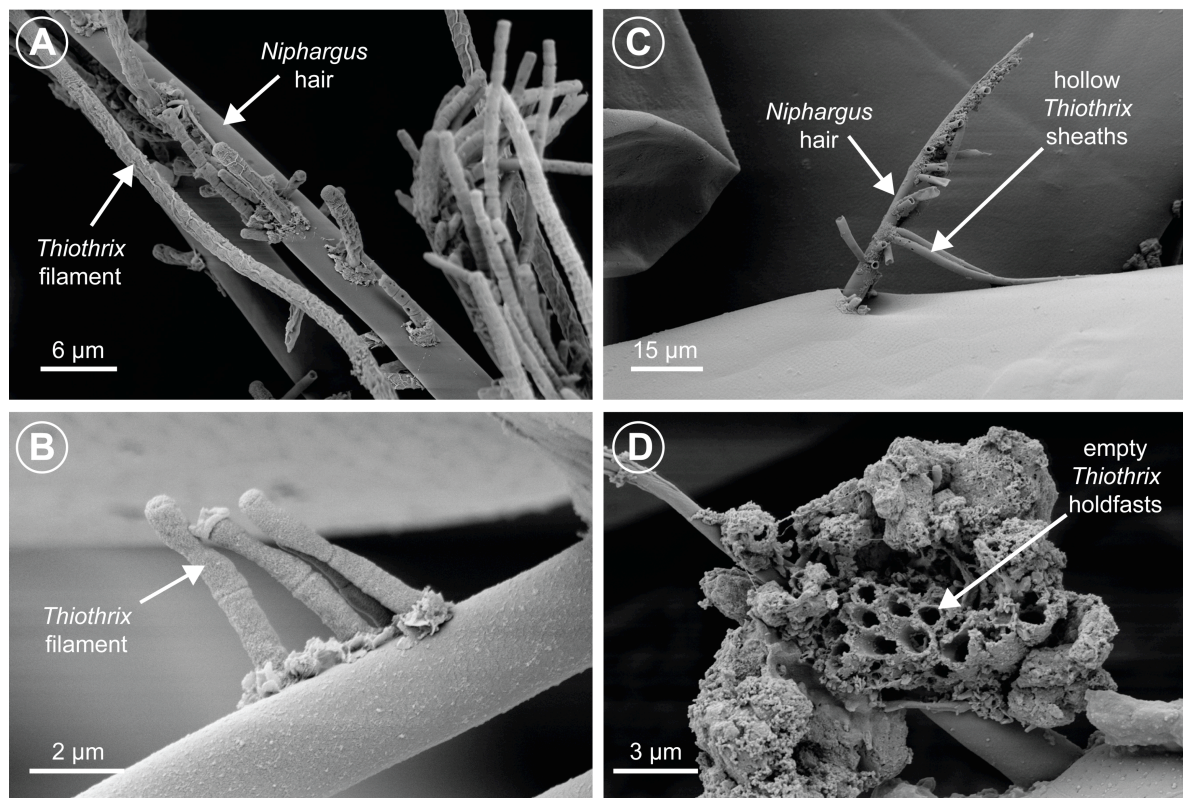


Figure 3.2. Scanning electron micrographs of intact filaments and remnants of *Thiothrix* ectosymbionts on *Niphargus*. (A) and (B): Intact ectosymbiotic *Thiothrix* filaments on *Niphargus* (images are of *Niphargus* individuals not subjected to the incubation experiment). (C) and (D): Empty *Thiothrix* sheaths and holdfasts on *Niphargus* individuals treated with streptomycin solution.

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SSS was last injected 14:00 h after the start of the experiment. Final sulfide concentrations between 13.0 and 16.3 mM and oxygen concentrations between 22 and 6 μM were measured in B1–B4. Even after adjusting the pH-values to ~ 8 , all antibiotically treated *N. ictus* individuals in B2 and all non-treated *N. frassianus* in B3 were found to be dead. Three non-treated *N. ictus* in B1 were still alive and rapidly fanning with their pleopods. Also, one antibiotically treated *N. frassianus* in B4 was still moving.

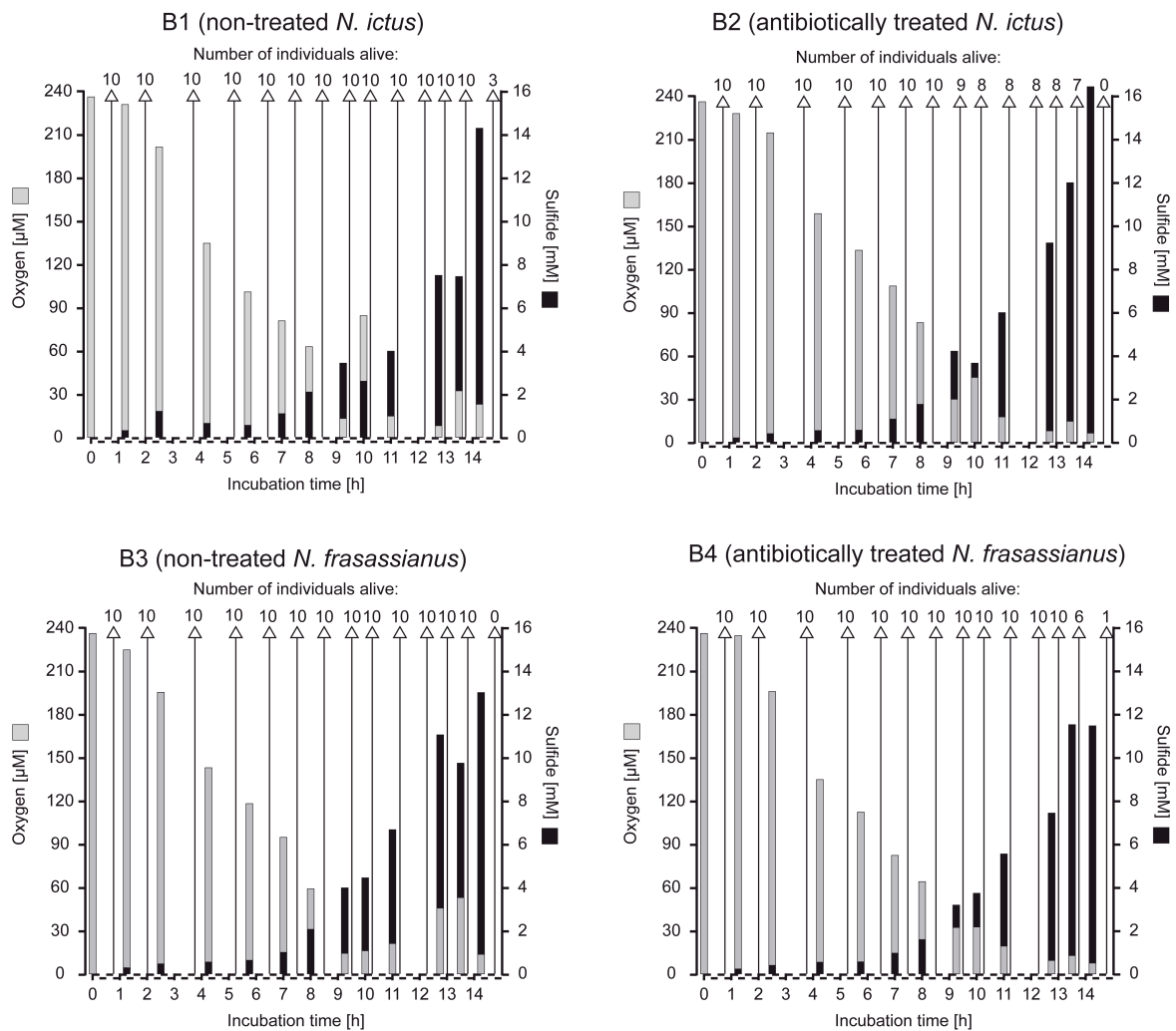


Figure 3.3. Bar charts showing oxygen and sulfide concentrations as well as survival rates of *Niphargus* in beakers B1–B4 over the time course of the incubation experiment.

22:00 h after the start of the experiment, the three surviving non-treated *N. ictus* in B1 were still alive, whereas the last *N. frassianus* individual in B4 had died. The five

control animals (one *N. ictus* and four *N. frasassianus*, all antibiotically treated; B5) had survived the whole experiment in non-sulfidic, filter-sterilized BG cave water.

3.5 Discussion

Frasassi-dwelling *Niphargus* have an exceptionally high sulfide tolerance

In Frasassi cave waters, *Niphargus* amphipods are exposed to sulfide concentrations of up to 0.5 mM (Galdenzi *et al.*, 2008). In our experiment, this concentration was exceeded in all incubation waters after the fourth injection of SSS (Figure 3.3; Supplementary Table S3.1), and all *Niphargus* individuals, with or without symbionts, survived far beyond this point. This suggests that *N. ictus* and *N. frasassianus* do not rely on their ectosymbionts to withstand sulfide in their natural habitat, but instead have physiological adaptations that allow for tolerating high sulfide and low oxygen levels.

Symbiont-independent sulfide detoxification processes have been studied in a few crustaceans (Vetter *et al.*, 1987; Vismann, 1991; Hagerman and Vismann, 1993; Johns *et al.*, 1997), and the Frasassi-dwelling *Niphargus* species may employ similar mechanisms. Crustaceans are commonly not able to exclude sulfide from their bodies, but they can oxidize it to non-toxic thiosulfate ($S_2O_3^{2-}$) or sulfite (SO_3^{2-}). Detoxification processes mainly take place in the hepatopancreas, either enzymatically mediated by sulfide oxidase (Vetter *et al.*, 1987) or via oxygen-independent sulfide binding to metallic ions (Vismann, 1991). Additionally, mitochondrial sulfide oxidation has been suggested to occur in the muscle tissue of some crustaceans (Vismann, 1991; Johns *et al.*, 1997). Hemocyanins are copper-containing proteins responsible for oxygen transport in the hemolymph of certain arthropods, including *Niphargus* (Gibert, 1971). As opposed to iron-containing hemoglobins, hemocyanins are likely not affected by the presence of sulfide (Childress *et al.*, 1987; Hagerman and Vismann, 1993). Transfer of sulfide to the hepatopancreas may therefore proceed without impeding oxygen transport mediated by hemocyanins.

High sulfide concentrations usually co-occur with low oxygen levels in the environment, as also seen in our experiment. If the available amount of oxygen is not sufficient to oxidize sulfide completely, several crustaceans can switch to an anaerobic

metabolism in order to protect cytochrome *c* oxidase from sulfide poisoning (Hagerman and Vismann, 1993; Johns *et al.*, 1997). Facultative anaerobiosis under hypoxic conditions has also been shown in two hypogean *Niphargus* species (Hervant *et al.*, 1995, 1996). Lactate, a common waste product accumulated by *Niphargus* upon anaerobic metabolism, may further act as a cofactor in increasing the oxygen affinity of hemocyanins (Truchot, 1980; Sanders and Childress, 1992; Hagerman and Vismann, 2001).

In this study, all 20 symbiont-bearing *N. ictus* and *N. frasassianus* individuals withstood sulfide concentrations of more than 7 mM. To the best of our knowledge, this is so far the highest experimentally determined sulfide concentration tolerated by a crustacean (Vaquer-Sunyer and Duarte, 2010). Amphipods usually show far lower sulfide tolerances (Theede *et al.*, 1969; Oseid and Smith, 1974a; Sandberg-Kilpi *et al.*, 1999). In a study similar to the present one, Knezovich *et al.* (1996) found that all 20 individuals of the infaunal amphipod *Rhepoxynius abronius* Barnard 1960 used for their experiment had died after 48h exposure to 78 μ M sulfide, and 14 out of 20 individuals of the infaunal amphipod *Eohaustorius estuarius* Bosworth 1973 had died after 48h exposure to 116 μ M sulfide. The evidence suggests that *Niphargus* from Frasassi are exceptional among amphipods with regard to their ability to live in highly sulfidic environments. Sulfide levels reported from the Frasassi caves are typically measured using water samples (Galdenzi *et al.*, 2008; Dattagupta *et al.*, 2009). However, Frasassi-dwelling *Niphargus* are often seen diving or crawling in black sediment underlying the cave waters. It is thus possible that the amphipods are exposed to much higher sulfide and lower oxygen levels in these sediments than measured in the water, and their high tolerance to both could enable them to successfully disperse among various cave water bodies via sediment-filled subsurface cracks and fissures.

Swimming behavior may have aided survival during the experiment

At the end of our experiment, three *N. ictus* individuals were still alive, whereas all *N. frasassianus* individuals had died almost 8 h before. Throughout the experiment, *N. ictus* and *N. frasassianus* individuals showed typical behavioral responses to hypoxia

(Burnett and Stickle, 2001). The crawling *N. frasassianus* remained at the bottom of the beakers. Not able to escape from the oxygen-depleted water, they were seen to minimize their locomotion, possibly in order to depress their metabolic rate. A similar reaction to hypoxia has been reported from two other hypogean *Niphargus* species (Hervant *et al.*, 1995, 1996). In contrast, *N. ictus* are active swimmers and could move to the air-water interface, where they were observed to stir the uppermost water layers by swift movements of their pereopods. This behavior presumably gave them access to more oxygen, and their ability to swim may have enabled *N. ictus* to survive longer than *N. frasassianus* during the experiment.

Limitations of this study

In this study, only acute sulfide toxicity levels were tested for *N. ictus* and *N. frasassianus*. It remains open if lower sulfide concentrations would have been lethal in case of longer exposure times. Also, sulfide values presented here refer to total sulfide concentrations. Hydrogen sulfide (H_2S) in aqueous solutions dissociates into hydrosulfide (HS^-) and bisulfide (S^{2-}), and the proportions of these three species vary with pH (Bagarinao, 1992). H_2S is the most toxic form of sulfide, as the unionized molecule readily crosses cell membranes, whereas ionized species are largely excluded. In the course of our experiment, pH-values of the incubation waters were periodically adjusted to ~ 8 after addition of SSS, as sodium sulfide has an alkalizing effect (Theede *et al.*, 1969). At pH=8, the ratio between H_2S and HS^- in the water is about 1:10 (Carroll, 1998). The proportion of H_2S in water increases as the pH decreases, so that H_2S and HS^- occur in almost equal shares at a pH-value slightly below 7. Frasassi cave waters have pH-values around 7.4 (Sarbu *et al.*, 2000). Thus, in their natural habitat, *N. ictus* and *N. frasassianus* may be sensitive to approximately 3 times lower total sulfide concentrations than those tolerated in our experiment (Carroll, 1998).

Frasassi cave waters are characterized by low and relatively stable temperatures (12.8–13.7 °C; Sarbu *et al.*, 2000). However, the temperature of the water in which the *Niphargus* were incubated during our experiment was between 20.3 and 22.6 °C (Supplementary Table S3.1). This discrepancy between cave water and experimental

temperatures could not be avoided due to the lack of a temperature-controlled room at the field station. The fact that several symbiont-free *Niphargus* died earlier than symbiont-bearing individuals in our experiment may have been due to the elevated incubation temperatures. A temperature rise of +6 °C induces substantial thermal stress in hypogean *Niphargus* (Colson-Proch *et al.*, 2010). This thermal stress may have been particularly detrimental for individuals that had been *a priori* weakened by antibiotic treatment.

3.6 Acknowledgements

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3.7 Supplementary Material

Supplementary Table S3.1. Overview of the course and results of the incubation experiment.
 SSS = sulfide stock solution. n.d. = not determined.

	Time [h:min]	Addition of SSS														End of experiment
		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	22:00	
Beaker B1 <i>N. ictus</i> non-treated					adjusted pH to ~8.0		adjusted pH to ~8.0	adjusted pH to ~7.6	adjusted pH to ~7.8	adjusted pH to ~8.0	adjusted pH to ~8.0	adjusted pH to ~8.0	adjusted pH to ~8.0	adjusted pH to ~8.0		
	Sulfide [μM]	0	275	1094	563	522	997	2056	3388	2531	3938	7422	7375	14250	n.d.	
	Oxygen [μM]	236	231	201	134	100	80	63	13	85	14	7	32	22	n.d.	
	pH-value	8.16	8.49	8.63	8.74	8.61	8.97	8.94	9.75	8.27	10.02	10.89	10.40	11.43	n.d.	
	Conductivity [$\mu\text{S/cm}$]	1499	1561	1586	1608	1650	1691	1776	2013	2047	2390	3002	3540	4550	n.d.	
	Temperature [$^{\circ}\text{C}$]	20.3	20.4	20.9	20.6	20.8	21.0	21.4	21.7	22.5	21.7	21.8	22.2	22.0	n.d.	
Number of individuals alive	10	10	10	10	10	10	10	10	10	10	10	10	10	3	3	
Beaker B2 <i>N. ictus</i> antibiotically treated																
	Sulfide [μM]	0	129	344	519	531	1088	1666	4169	3641	5938	9156	11969	16313	n.d.	
	Oxygen [μM]	236	227	214	158	133	108	82	29	45	17	7	14	6	n.d.	
	pH-value	8.16	8.45	8.64	8.70	8.57	8.92	9.13	10.08	9.59	10.30	10.84	10.89	11.31	n.d.	
	Conductivity [$\mu\text{S/cm}$]	1499	1544	1563	1586	1625	1663	1770	2034	2165	2550	3100	3500	4560	n.d.	
	Temperature [$^{\circ}\text{C}$]	20.3	20.4	21.2	20.6	20.8	21.1	21.5	21.7	22.7	21.7	22.0	21.9	22.0	n.d.	
Number of individuals alive	10	10	10	10	10	10	10	10	9	8	8	8	7	0	0	
Beaker B3 <i>N. fraassianus</i> non-treated																
	Sulfide [μM]	0	255	466	494	641	975	1997	3906	4422	6625	11047	9750	13000	n.d.	
	Oxygen [μM]	236	224	195	143	117	94	58	14	16	20	47	52	13	n.d.	
	pH-value	8.16	8.45	8.58	8.59	8.50	8.91	8.93	9.93	10.34	10.37	10.40	9.81	11.16	n.d.	
	Conductivity [$\mu\text{S/cm}$]	1499	1539	1556	1573	1611	1651	1711	1992	2330	2700	2960	3420	4470	n.d.	
	Temperature [$^{\circ}\text{C}$]	20.3	20.5	21.3	20.7	20.8	21.0	21.3	21.7	22.4	21.7	22.2	22.2	22.2	n.d.	
Number of individuals alive	10	10	10	10	10	10	10	10	10	10	10	10	10	0	0	
Beaker B4 <i>N. fraassianus</i> antibiotically treated																
	Sulfide [μM]	0	214	399	538	538	900	1534	3156	3719	5531	7422	11531	11438	n.d.	
	Oxygen [μM]	236	234	195	135	112	82	64	32	33	19	10	12	9	n.d.	
	pH-value	8.16	8.41	8.65	8.73	8.58	8.84	9.07	9.83	9.66	10.15	10.68	10.65	11.19	n.d.	
	Conductivity [$\mu\text{S/cm}$]	1499	1535	1563	1586	1633	1664	1759	1916	2210	2560	3090	3620	4720	n.d.	
	Temperature [$^{\circ}\text{C}$]	20.3	20.4	21.3	20.8	20.9	21.1	21.5	21.7	22.2	21.7	22.0	22.0	22.6	n.d.	
Number of individuals alive	10	10	10	10	10	10	10	10	10	10	10	6	1	0	0	
Beaker B5 (Controls) antibiotically treated																
	Sulfide [μM]	0	0	0	0	0	0	0	0	0	0	0	0	0	n.d.	
	Oxygen [μM]	236	254	253	235	252	249	247	246	247	242	239	238	237	n.d.	
	pH-value	8.16	8.08	8.06	8.05	8.00	8.01	7.97	7.98	7.96	7.96	7.97	7.94	7.87	n.d.	
	Conductivity [$\mu\text{S/cm}$]	1499	1528	1521	1527	1534	1529	1527	1527	1531	1528	1530	1538	1543	n.d.	
	Temperature [$^{\circ}\text{C}$]	20.3	20.3	20.8	20.6	20.9	21.1	21.4	21.5	21.9	21.2	21.2	21.2	21.3	n.d.	
Number of individuals alive	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	1+4	1+4	

Chapter 4:

Niphargid-*Thiothrix* ectosymbioses are widespread in sulfidic groundwater ecosystems: evidence from Romania

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4.1 Abstract

Sulfide is a metabolic poison that inhibits respiration. Nevertheless, niphargid groundwater amphipods have been reported to thrive in the sulfide-rich chemoautotrophic environments of the Frasassi caves in Italy and Movile cave in Romania. Following the recent discovery that four species of *Niphargus* co-occur in Frasassi and that at least three of them harbor ectosymbiotic, sulfide-oxidizing bacteria of the genus *Thiothrix* (falling into the clades T1–T3), we used molecular tools to investigate the biodiversity of *Niphargus* and *Pontoniphargus* amphipods in Movile cave and the surrounding Dobrogea region (comprising both sulfidic and non-sulfidic environments) and to examine them for *Thiothrix* ectosymbionts. Phylogenetic and haploweb analyses of three molecular markers (mitochondrial COI and nuclear 28S and ITS) in 70 specimens revealed the presence of two *Pontoniphargus* and five *Niphargus* species, which were confirmed morphologically. Four species (*Niphargus* cf. *stygius*, *Niphargus hrabei*, *Pontoniphargus racovitzai*, and *Pontoniphargus ruffoi*) occurred in sulfidic waters and the three others (*Niphargus decui*, *Niphargus dobrogeicus*, and *Niphargus gallicus*) solely in non-sulfidic areas. The two *Pontoniphargus* species were firmly nested within the genus *Niphargus*, suggesting that this generic distinction is unwarranted. PCR screens detected *Thiothrix*-like 16S sequences in niphargid DNA extracts from six of the seven species, and the presence of *Thiothrix* filaments on the cuticle of five niphargid individuals was confirmed by SEM. Two main clades of *Thiothrix* bacteria were detected: one of them occurred only on specimens collected in sulfidic locations, whereas the other one was found on niphargids from both sulfidic and non-sulfidic areas (some specimens harbored both clades). Although Frasassi and Dobrogean niphargids are not closely related in the *Niphargus* phylogeny, the first Dobrogean *Thiothrix* group (named T4) is closely related to T1 and T2 Frasassi ectosymbionts, whereas the second group is indistinguishable from Frasassi T3 ectosymbionts. This striking parallel between chemoautotrophic ecosystems 1200 km apart has profound implications regarding the origin and evolution of *Niphargid-Thiothrix* ectosymbioses.

4.2 Introduction

Since their discovery at hydrothermal vents in the late 1970s, myriad examples of symbioses between chemoautotrophic, sulfide-oxidizing bacteria and invertebrates have been found worldwide in sulfidic marine environments (Dubilier *et al.*, 2008). Dark, isolated, and sulfide-rich habitats analogous to hydrothermal vents also exist in land-locked freshwater caves, such as the Frasassi caves in Italy and Movile cave in Romania (Forti *et al.*, 2002). Recently, ectosymbioses between sulfur-oxidizing *Thiothrix* bacteria and three species of the groundwater amphipod genus *Niphargus* were reported from the Frasassi caves (Dattagupta *et al.*, 2009; Bauermeister *et al.*, 2012), extending the realm of chemosynthetic symbioses into freshwater ecosystems.

The three ectosymbiont-bearing *Niphargus* amphipod species in Frasassi are *N. ictus*, *N. frasassianus*, and *N. montanarius*, and they harbor three distinct clades (T1–T3) of *Thiothrix* ectosymbionts on their cuticles (Bauermeister *et al.*, 2012). While clade T1 is only present on *N. frasassianus*, a species restricted to sulfidic waters, clades T2 and T3 are found on *Niphargus* species in both sulfidic and non-sulfidic locations (T2 on *N. ictus* and *N. montanarius*, and T3 on all three Frasassi-dwelling species). These three ectosymbiont clades do not form a monophyletic group (Bauermeister *et al.*, 2012), and neither do their host species (Flot *et al.*, 2010a). The lack of congruence between the host and symbiont phylogenies suggests independent establishments of the symbioses and/or interspecies symbiont transfer.

Niphargus is the largest freshwater amphipod genus in the world, consisting of over 300 species spread over most of Europe (Väinölä *et al.*, 2008). Although sulfide is a potent inhibitor of mitochondrial electron transfer (Bagarinao, 1992) and generally toxic to aquatic life (Theede *et al.*, 1969; Oseid and Smith, 1974a; Sandberg-Kilpi *et al.*, 1999), several niphargid amphipods of the *Niphargus* and closely related *Pontoniphargus* genera thrive in sulfide-rich environments. Well-documented examples include not only *N. ictus* and *N. frasassianus* in Frasassi (up to 415 μM sulfide; Flot *et al.*, 2010a), but also *N. stefanelli* in Acquapuzza, Italy (410 μM sulfide; Latella *et al.*, 1999), and *N. cf. stygius*, *P. racovitzai*, and *P. ruffoi* in Movile cave, Romania (about 500 μM sulfide; Sarbu, 2000). Two further species, *N. hebereri* and *N. pectencoronatae*, are found in anchihaline caves in Croatia, where sulfide has been reported but not yet been quantified (Sket, 1996; Gottstein *et al.*, 2007).

Since the studies from the Frasassi caves suggested that the *Niphargus-Thiothrix* ectosymbiosis likely evolved independently multiple times, we hypothesized that this association would also be found in niphargids from other sulfidic groundwater habitats. The Dobrogea region of south-western Romania provided the ideal locality to test this hypothesis, as it contains a sulfidic groundwater aquifer that can be accessed through man-made wells, springs, and also Movile cave. Discovered in 1986, Movile cave was the first terrestrial chemoautotrophic ecosystem described (Sarbu and Popa, 1992; Sarbu *et al.*, 1996; Sarbu, 2000) and is one of the most thoroughly studied to date together with Frasassi. The cave is known to harbor three niphargid species, and amphipods have also been previously reported from both sulfidic and non-sulfidic wells and springs in the surrounding region. Our goals in this study were two-fold: first, to molecularly characterize the niphargids in the Dobrogea region and compare them phylogenetically with Frasassi-dwelling species, and second, to examine them for the presence of *Thiothrix* ectosymbionts using microscopy and molecular methods.

4.3 Materials and Methods

Collection and provision of samples

By means of aspiration devices, niphargid amphipods were collected from Movile cave and from springs and wells in the Dobrogea region (Figure 4.1). Samples were preserved in 70% ethanol (for DNA sequence analyses and morphological examination), in RNA later (for DNA sequence analyses), and in 2.5% glutaraldehyde prepared in Tris buffer (for scanning electron microscopy). DNA extracted from a Movile cave microbial mat sample was provided by Colin Murrell and Jason Stephenson (University of Warwick, UK). The mat sample had been collected from airbell 2 in Movile and kept frozen at -20 °C until DNA extraction using a method described in Neufeld *et al.* (2007).

Host sequence analyses

A total of 72 specimens were analyzed molecularly (Table 4.1): 70 niphargids and 2 outgroups (*Synurella* sp. and *Gammarus* sp.). DNA extractions, PCR amplifications, and sequencing of the mitochondrial COI and the nuclear ITS and 28S were performed as

reported previously (Flot *et al.*, 2010a). The sequences of length-variant heterozygotes (Flot *et al.*, 2006) were unravelled using the online program Champuru (Flot, 2007; <http://www.mnhn.fr/jfflot/champuru>), and other heterozygotes were phased using Clark's method (Clark, 1990).

Sequences were aligned by eye in MEGA5 (Kumar *et al.*, 2008) for COI or using MAFFT's E-INS-i option (Kato *et al.*, 2002) for 28S and ITS. Maximum-likelihood phylogenetic analyses were performed in MEGA5 under the GTR+G+I model (using all sites) and with 1000 bootstrap replicates (Felsenstein, 1978). The ITS and 28S phylogenetic trees were turned into haplowebs by adding connections between haplotypes found co-occurring in heterozygous individuals (Flot *et al.*, 2010b; Flot *et al.*, 2011).



Figure 4.1. Distribution of sampling sites in the Dobrogea region. Most sampling locations (black dots) were located in the vicinity of the town of Mangalia in the Romanian part of Dobrogea (including Movile cave, just a few kilometers west of Mangalia). In addition, one *Niphargus decui* individual was collected in the town of Shabla in the Bulgarian part of Dobrogea.

Chapter 4 – Niphargid-*Thiothrix* ectosymbioses in Romania

Table 4.1. List of amphipod specimens analyzed molecularly in this study.

Code	Niphargid species	Town	Location	<i>Thiothrix</i> epibiont
AH_10.1	<i>P. racovitzai</i>	Mangalia	Mobile Cave	-
AH_10.2	<i>P. ruffoi</i>	Hagieni		T4
AH_10.3	<i>P. ruffoi</i>	Hagieni		T4
AH_10.4	<i>P. ruffoi</i>	Hagieni		T4
AH_10.5	<i>P. ruffoi</i>	Hagieni		T4
AH_10.11	<i>P. racovitzai</i>	Mangalia	Mobile Cave	-
AH_10.12	<i>N. cf. stygius</i>	Mangalia	Mobile Cave	-
AH_12.1	<i>P. racovitzai</i>	Mangalia	Mobile Cave	-
AH_12.2	<i>Synurella ambulans</i>	Hagieni		-
AH_12.3	<i>N. gallicus</i>	Dulcești		other
AH_12.4	<i>N. decui</i>	Dulcești		-
AH_12.5	<i>N. decui</i>	Vama Veche		-
AH_12.6	<i>N. decui</i>	Dulcești		-
CF_09.21	<i>P. racovitzai</i>	Mangalia	str. Pictor Tonitza #1	T4
CF_09.22	<i>P. racovitzai</i>	Mangalia	str. Pictor Tonitza #1	-
CF_09.24	<i>N. cf. stygius</i>	Mangalia	str. Gheorge Netoi #1A	T4
CF_09.25	<i>N. cf. stygius</i>	Mangalia	str. Gheorge Netoi #1A	-
JFF_12.3	<i>N. cf. stygius</i>	Mangalia	str. Pictor Tonitza #1	-
JFF_12.4	<i>N. cf. stygius</i>	Mangalia	str. Pictor Tonitza #1	-
JFF_12.6	<i>P. racovitzai</i>	Mangalia	str. Pictor Tonitza #1	-
JFF_12.13	<i>Gammarus</i> sp.	Pescineaga		-
JFF_12.18	<i>P. racovitzai</i>	Mangalia	str. Pictor Tonitza #1	-
JFF_12.19	<i>P. racovitzai</i>	Mangalia	str. Pictor Tonitza #1	T4
JFF_12.25	<i>N. decui</i>	Mangalia	str. Pictor Tonitza #1	-
JFF_12.28	<i>N. decui</i>	Mangalia	str. Matei Basarab #74	-
JFF_12.29	<i>N. hrabei</i>	Hagieni		-
JFF_12.31	<i>N. cf. stygius</i>	Mangalia	str. Ion Mecu #51	T3
JFF_12.32	<i>N. cf. stygius</i>	Mangalia	str. Ion Mecu #51	T4
JFF_12.36	<i>P. racovitzai</i>	Mangalia	Mobile Cave	-
JFF_12.39	<i>N. cf. stygius</i>	Mangalia	Mobile Cave	T3+T4
JFF_12.42	<i>N. decui</i>	Shabla	Magazin Alim	-
JFF_12.43	<i>N. decui</i>	Mangalia	str. Horia Cloșca Crișan #13	-
JFF_12.44	<i>N. gallicus</i>	Mangalia	str. Vasile Pârvan #16	-
JFF_12.45	<i>N. cf. stygius</i>	Mangalia	Aleea Cetății #1	-
JFF_12.46	<i>P. racovitzai</i>	Mangalia	Aleea Cetății #1	T3
JFF_12.50	<i>N. cf. stygius</i>	Mangalia	str. Matei Basarab #62	T4
JFF_12.52	<i>N. decui</i>	Mangalia	str. Matei Basarab #62	-
SS_10.1	<i>N. cf. stygius</i>	Mangalia	str. Dumitru Ana #13	T3+T4
SS_10.2	<i>N. cf. stygius</i>	Mangalia	str. Dumitru Ana #13	T3+T4
SS_10.3	<i>N. decui</i>	Albești		-
SS_10.4	<i>N. decui</i>	Albești		-
SS_10.5	<i>N. dobrogicus</i>	Vama Veche		-
SS_10.6	<i>N. decui</i>	Vama Veche		-
SS_10.7	<i>N. dobrogicus</i>	Doi Mai		T3
SS_10.8	<i>N. decui</i>	Doi Mai		T3
SS_11.1	<i>N. cf. stygius</i>	Mangalia	Mobile Cave	T3
SS_11.3	<i>N. decui</i>	Mangalia	str. Mihai Viteazul 20	-
SS_11.4	<i>N. decui</i>	Albești	well #20	-
SS_11.5	<i>N. decui</i>	Albești	PD well	-
SS_11.6	<i>N. hrabei</i>	Hagieni		other
SS_11.7	<i>N. gallicus</i>	Dulcești		-
SS_11.8	<i>N. dobrogicus</i>	Doi Mai	str. Mihail Kogălniceanu 474	-
SS_11.9	<i>N. decui</i>	Doi Mai	str. Mihail Kogălniceanu 474	-
SS_11.10	<i>N. dobrogicus</i>	Doi Mai	str. Mihail Kogălniceanu 393	-
SS_11.11	<i>N. decui</i>	Doi Mai	str. Mihail Kogălniceanu 393	-
SS_11.12	<i>N. decui</i>	Limanu	str. Vuia str/ str. Crinului	-
SS_11.13	<i>N. decui</i>	Limanu	G well	-
SS_11.14	<i>N. decui</i>	Limanu	G well	-
SS_11.15	<i>N. dobrogicus</i>	Limanu	G well	-
SS_11.16	<i>N. decui</i>	Limanu	F well	-
SS_11.17	<i>N. decui</i>	Limanu	G2 well	-
SS_11.18	<i>N. hrabei</i>	Hagieni		-
SS_11.19	<i>N. cf. stygius</i>	Mangalia	Mobile Cave	-
SS_11.20	<i>N. cf. stygius</i>	Mangalia	Mobile Cave	-
SS_11.21	<i>N. cf. stygius</i>	Mangalia	Mobile Cave	-
TB_11.1	<i>N. decui</i>	Limanu	H2 well	-
TB_11.2	<i>N. decui</i>	Limanu	H2 well	-
TB_11.3	<i>N. decui</i>	Limanu	H2 well	-
TB_11.6	<i>N. gallicus</i>	Mangalia	str. Delfinului 16	T3
TB_12.1	<i>N. gallicus</i>	Mangalia	str. Delfinului 10	-
TB_12.2	<i>N. gallicus</i>	Mangalia	str. Delfinului 10	-
TB_12.3	<i>N. gallicus</i>	Mangalia	str. Maior Giurescu 22	other

Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was used to search for *Thiothrix* epibionts on six *P. racovitzai* specimens (three from sulfidic water inside Movile cave and three from non-sulfidic wells in the town of Mangalia) and four *N. cf. stygius* individuals (two from Movile cave, one from a sulfidic well in Mangalia, and one from a non-sulfidic well in the same town). Sample preparation and analysis were done as described previously (Bauermeister *et al.*, 2012).

Design and optimization of *Thiothrix*-specific primers

We used the *Thiothrix*-specific forward primer THIO714F (5'-ATG CAT AGA GAT CGG AAG G-3'; Bauermeister *et al.*, 2012) and the newly designed reverse primer THIO1492R (5'-GGC TAC CTT GTT ACG ACT T-3') for construction of 16S rDNA clone libraries and for direct PCR screenings. THIO1492R was designed and tested for matching with publicly available *Thiothrix* sequences using PRIMROSE (Ashelford *et al.*, 2002). Gradient PCRs were performed to determine the optimum annealing temperature for the primer pair. THIO714F worked well for PCRs, but not for sequencing. Therefore, another *Thiothrix*-specific forward primer (THIO718Fseq; 5'-ATA GAG ATC GGA AGG AAC A-3') was designed as described above and used in combination with THIO1492R for direct sequencing of PCR products.

Thiothrix clone library construction

Partial 16S rDNA clone libraries were obtained from one *P. ruffoi* sample (AH_10.4), one *N. cf. stygius* sample (SS_10.1), and the Movile microbial mat sample. PCR mixtures (50 μ L) contained 1x ammonium buffer (Bioline, Luckenwalde, Germany), 2 mM MgCl₂ (Bioline), 0.3 mM dNTP mix (Bioline), 25–30 ng of DNA (quantified by a ND-1000 Nanodrop, PEQLAB Biotechnology, Erlangen, Germany), 1.25 units of BioTaq DNA polymerase (Bioline), and 25 pM each of the primers THIO714F and THIO1492R. PCR was performed in a SensoQuest LabCycler (SensoQuest, Göttingen, Germany), with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 56 °C for 25 s, 72 °C for 2.5 min, and a final extension at 72 °C for 5 min. PCR products were checked on a 1% agarose gel. Bands of the expected size were excised and

extracted using the QIAquick Gel Extraction Kit (QIAGEN). 16S rDNA fragments were cloned and sequenced as described previously (Bauermeister *et al.*, 2012). Sequences were manually checked for ambiguities using CodonCode Aligner version 3.7.1.2 (CodonCode Corporation, Dedham, MA, USA), and screened for chimeras using Pintail version 1.0 (Ashelford *et al.*, 2005). Putative chimeras were excluded from subsequent analyses.

PCR screenings

Extracts obtained from 72 amphipods (70 niphargids and two outgroups) were PCR-screened for *Thiothrix* DNA. PCR mixtures (10 μ L) contained the same ingredients as described for partial 16S clone library construction. PCR cycling conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 50 cycles of 94 °C for 45 s, 56 °C for 30 s, and 72 °C for 1.5 min. PCR products were checked on a 1% agarose gel. Samples revealing bands of the expected size were sequenced directly using the primers THIO718Fseq and THIO1492R. Sequences were assembled, manually checked for ambiguities, and screened for chimeras as described in the previous section.

Phylogenetic analysis of *Thiothrix* partial 16S rRNA gene sequences

Sequences obtained from clone libraries and PCR screenings were compared to sequences in the public GenBank database using nucleotide BLAST (Altschul *et al.*, 1990). A majority of the sequences (60 out of 68 obtained from clone libraries and 24 out of 36 obtained by direct PCR) was found to be closely related to sequences of cultivated *Thiothrix* species and to sequences previously obtained from *Niphargus* species and *Thiothrix*-dominated microbial mats in the Frasassi caves. 82 of these 84 sequences (leaving out two sequences obtained twice from PCR screens and clone libraries) were used for phylogenetic analyses together with 126 closely related *Thiothrix* sequences downloaded from GenBank. All sequences were aligned using the MAFFT version 6 multiple sequence alignment tool (Katoh and Toh, 2010) with the Q-INS-I strategy for consideration of RNA secondary structure (Katoh and Toh, 2008). The alignment was manually refined, and a 50% consensus filter was applied in

MOTHUR (Schloss *et al.*, 2009), resulting in 741 nucleotide positions used for phylogenetic analysis. jModelTest version 0.1.1 (Posada, 2008) was used to determine the best-suited nucleotide model among 88 possible models following the Bayesian Information Criterion. The selected model (TIM3+I+G) was used to build a Maximum Likelihood (ML) phylogenetic tree (1000 bootstrap replicates) using PhyML 3.0 (Guindon and Gascuel, 2003). The ML tree was rooted with an epibiont clone sequence from the hydrothermal vent galatheid crab *Shinkaia crosnieri* (GenBank accession number AB476284; Watsuji *et al.*, 2010). In addition, Neighbor-Joining (NJ) bootstrap values for all nodes were calculated based on the same alignment using the BioNJ algorithm (Kimura 2-parameter model; 1000 bootstrap replicates) implemented in SeaView version 4 (Gouy *et al.*, 2010).

4.4 Results

Delimitation of host species

All 72 specimens yielded 28S sequences, but sequencing the COI marker failed for five of them. ITS chromatograms were difficult to interpret for some species that had lots of intragenomic polymorphism, hence only the chromatograms that could be interpreted were included in the present study. Integration of nuclear and mitochondrial data with morphological features revealed the presence of nine niphargid species in our dataset: *Gammarus* sp., *Niphargus decui*, *Niphargus dobrogicus*, *Niphargus gallicus*, *Niphargus hrabei*, *Niphargus* cf. *stygius*, *Pontoniphargus racovitzai*, *Pontoniphargus ruffoi*, and *Synurella* sp. Only the COI marker distinguished all of them (Figure 4.2). The two *Pontoniphargus* species *P. racovitzai* and *P. ruffoi* were indistinguishable using 28S, as they shared the same sequences for this marker (Figure 4.3), but their monophyly was strongly supported by COI. Among the five *Niphargus* species, of which multiple specimens were analyzed, *N. decui* and *N. dobrogicus* were monophyletic for all markers analyzed, whereas the monophyly of *N. hrabei*, *N. gallicus*, and *N. cf. stygius* was only supported by COI and 28S (since these species were not included in the ITS tree, see Figure 4.4).

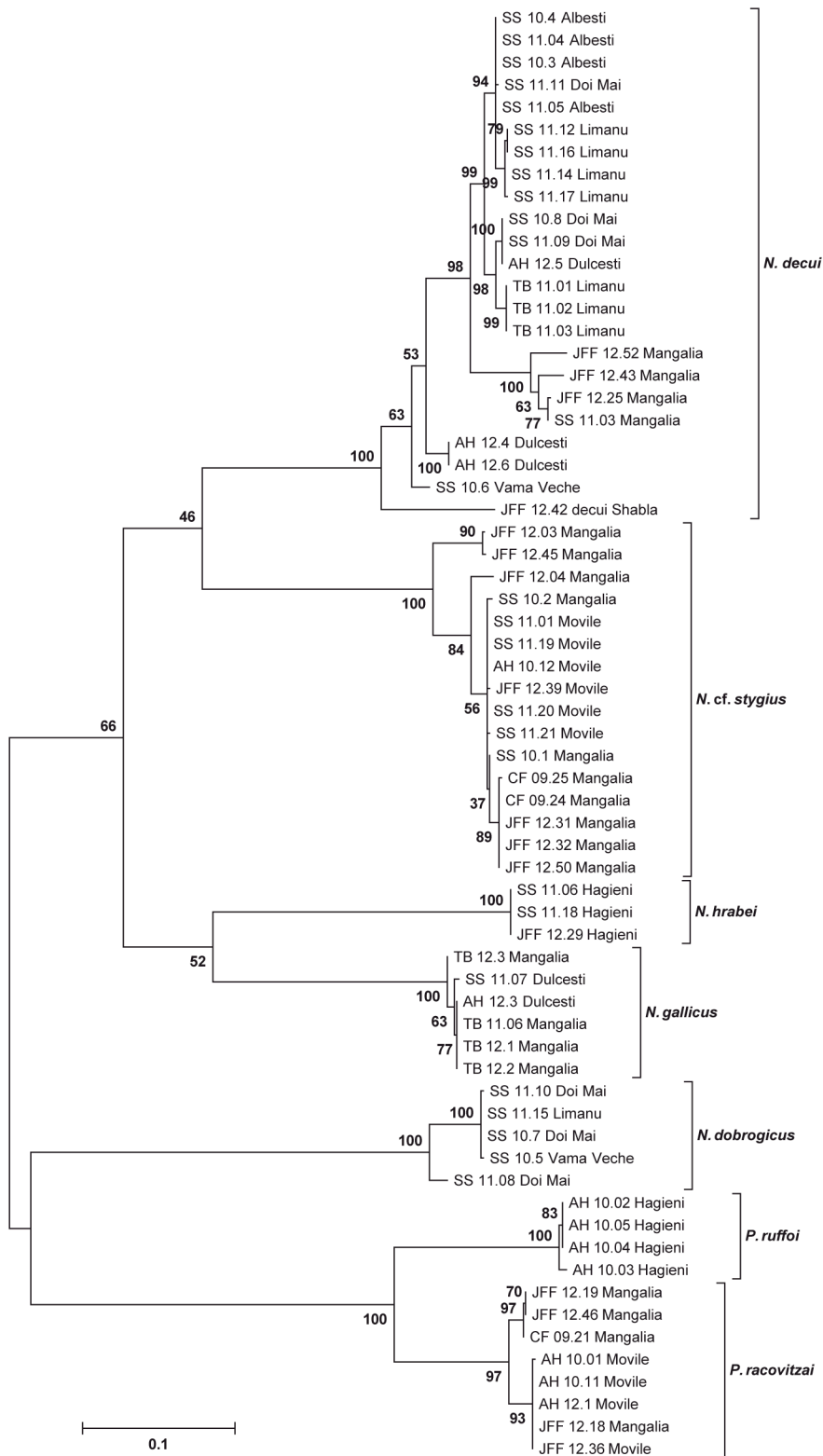


Figure 4.2. Maximum-likelihood COI phylogeny of niphargids collected in Dobrogea. The model used was GTR+G+I. Bootstrap values from 1000 replicates are shown on the nodes, and the names of the seven species identified are shown on the right side of each clade.

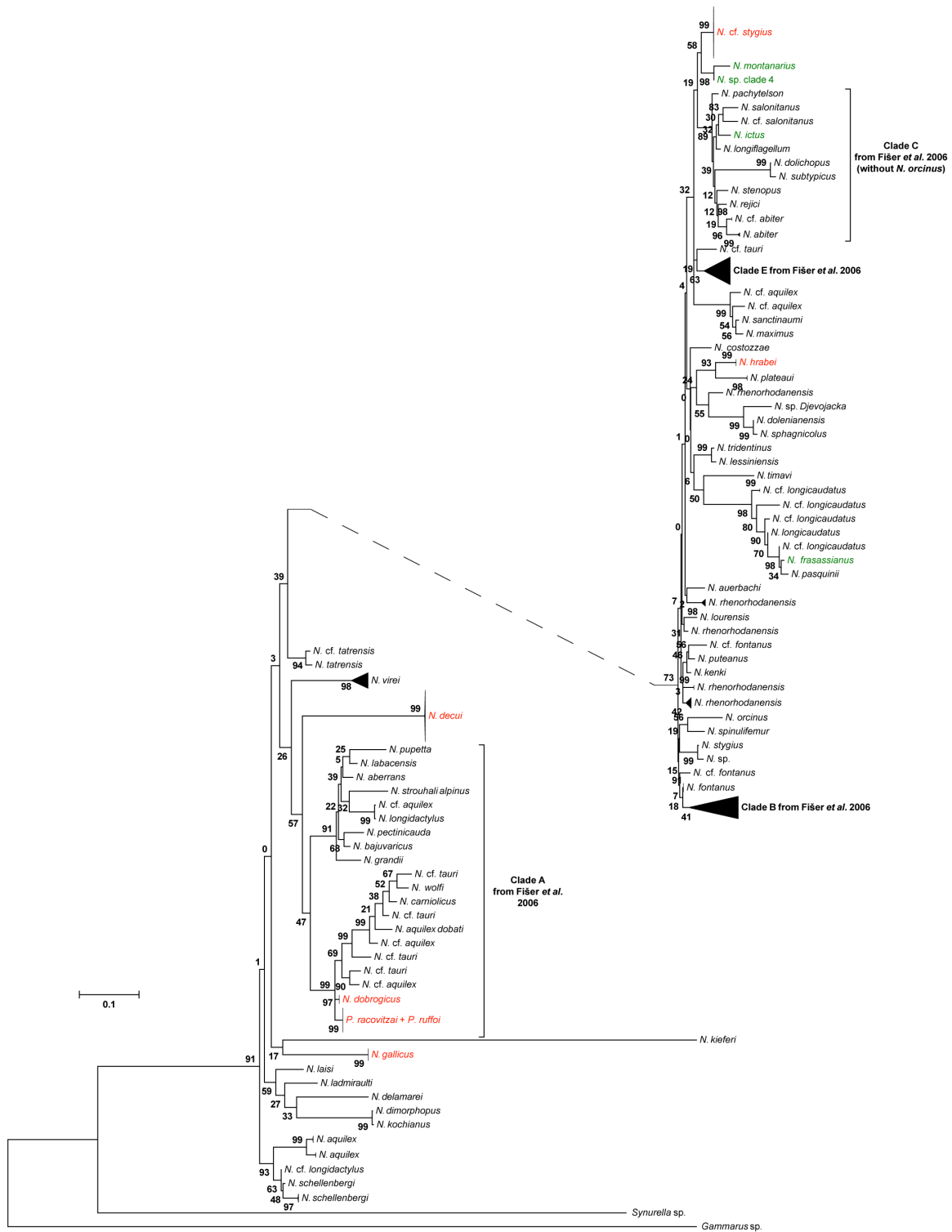


Figure 4.3. Maximum-likelihood 28S phylogeny of niphargid amphipods. The phylogeny was obtained under the GTR+G+I model with 1000 bootstrap replicates. It includes sequences from Lefébure *et al.* (2006), Lefébure *et al.* (2007), Fišer *et al.* (2008), Trontelj *et al.* (2009), Flot (2010), Flot *et al.* (2010a), and Hartke *et al.* (2011). Niphargid sequences from the present study are shown in red, sequences from Frasassi-dwelling *Niphargus* in green.

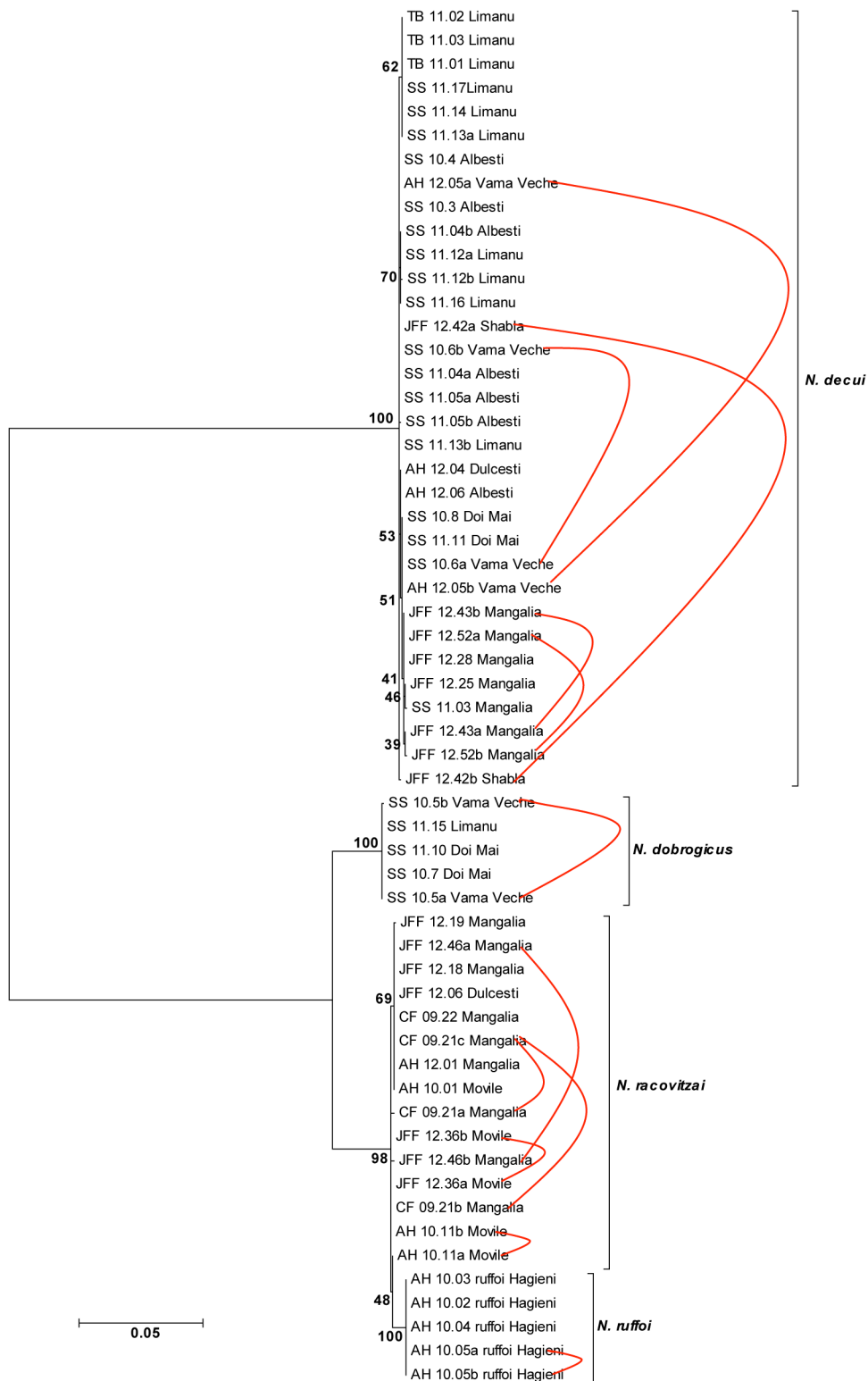


Figure 4.4. Haploneb of ITS sequences of four niphargid species. The haploneb was constructed by adding curves connecting the sequences found co-occurring in heterozygous individuals. The underlying phylogeny was obtained using a maximum-likelihood approach (model: GTR+G+I) with 1000 bootstrap replicates.

Phylogeny of the genus *Niphargus*

Our 28S phylogeny (Figure 4.3) does not support the putative sister-genus relationship between *Niphargus* and *Pontoniphargus*; on the contrary, *P. ruffoi* and *P. racovitzai* are placed with high support within *Niphargus* clade A (Fišer *et al.*, 2008), a clade characterized by a very high morphological variability encompassing at least five of the 13 *Niphargus* morphogroups originally defined by Straškraba (1972).

Detection of *Thiothrix* epibionts

Scanning electron microscopy (SEM) of niphargid specimens revealed *Thiothrix*-looking filaments on two out of three examined *P. racovitzai* specimens from Movile cave, on both investigated *N. cf. stygius* individuals from the same location, and on one *N. cf. stygius* specimen from a sulfidic well in Mangalia (Table 4.2). The filaments were predominantly attached to hairs and spines of the niphargids' appendages (Figure 4.5), similar to what has previously been shown for *Thiothrix* epibionts on *Niphargus* species from the Frasassi caves (Dattagupta *et al.*, 2009; Bauermeister *et al.*, 2012). *Thiothrix*-looking filaments were not observed on the three *P. racovitzai* specimens and the one *N. cf. stygius* individual from non-sulfidic wells, neither on one *P. racovitzai* specimen from Movile cave.

Table 4.2. List of niphargid specimens examined using SEM.

Code	Niphargid species	Town	Location	<i>Thiothrix</i> -looking filaments
AH_10.2	<i>P. racovitzai</i>	Mangalia	Movile Cave	x
AH_10.9	<i>P. racovitzai</i>	Mangalia	Movile Cave	x
JFF_12.20	<i>P. racovitzai</i>	Mangalia	str. Pictor Tonitza #1	
JFF_12.21	<i>P. racovitzai</i>	Mangalia	str. Pictor Tonitza #1	
JFF_12.26	<i>N. cf. stygius</i>	Mangalia	str. Pictor Tonitza #1	
JFF_12.33	<i>N. cf. stygius</i>	Mangalia	str. Ion Mecu #51	x
JFF_12.37	<i>P. racovitzai</i>	Mangalia	Movile Cave	
JFF_12.40	<i>N. cf. stygius</i>	Mangalia	Movile Cave	x
JFF_12.48	<i>P. racovitzai</i>	Mangalia	Aleea Cetății #1	
SS_10.10	<i>N. cf. stygius</i>	Mangalia	Movile Cave	x

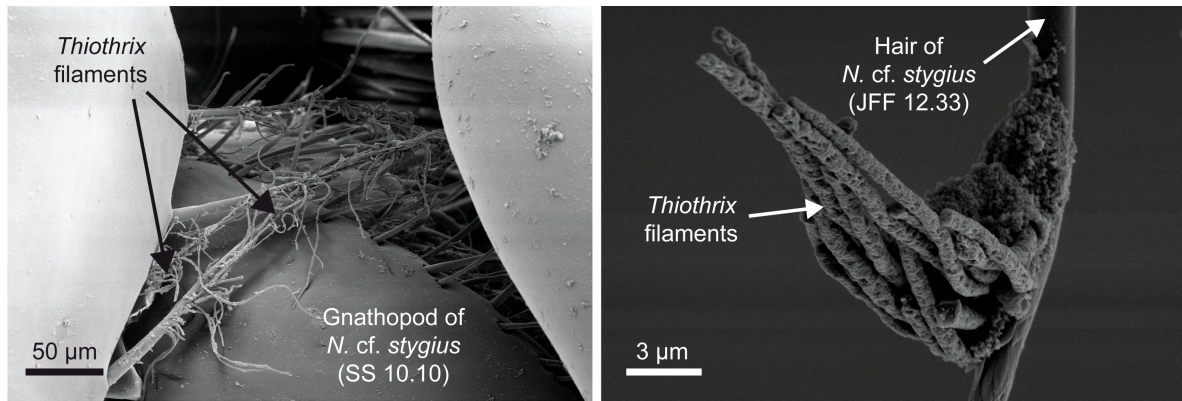


Figure 4.5. SEM images of *Thiothrix*-looking filaments attached to hairs on the appendages of *N. cf. stygius* from Movile cave (left) and from str. Ion Mecu #51 (right).

Thiothrix-related partial 16S rDNA sequences were obtained from 21 out of 72 niphargid DNA extracts using PCR with *Thiothrix*-specific primers. From three of these 21 DNA extracts, we obtained mixtures of two overlapping *Thiothrix* sequences. We were able to identify the individual sequences by comparison with *Thiothrix* sequences obtained from the clone libraries. From 14 of the remaining 51 niphargid samples, bands of the correct size were obtained on the agarose gel, but the corresponding sequences belonged to bacteria other than *Thiothrix* (Table 4.1).

Phylogeny of *Thiothrix* epibionts

The majority of *Thiothrix* 16S sequences obtained from niphargids by clone library construction and PCR screenings (87%) clustered into two different phylogenetic clades (T3 and T4; Figure 4.6). Clade T4, supported by a 88% ML bootstrap value, contained only sequences obtained from niphargids in sulfide-rich locations in Dobrogea: Hagieni, Movile cave, and two sulfidic wells located in the western part of Mangalia (Table 4.3). Clade T3 (95% ML bootstrap support) contained *Thiothrix* sequences obtained from specimens from both sulfidic and non-sulfidic areas. Furthermore, the same clade contained sequences of *Thiothrix* ectosymbionts shared among *N. frassianus* and *N. montanarius* from the Frasassi caves (Bauermeister *et al.*, 2012). All other *Thiothrix* epibiont sequences (13%), that did not fall within clade T3 or T4, clustered together with *Thiothrix* sequences obtained from Movile or Frasassi microbial mats.

Chapter 4 – Niphargid-*Thiothrix* ectosymbioses in Romania

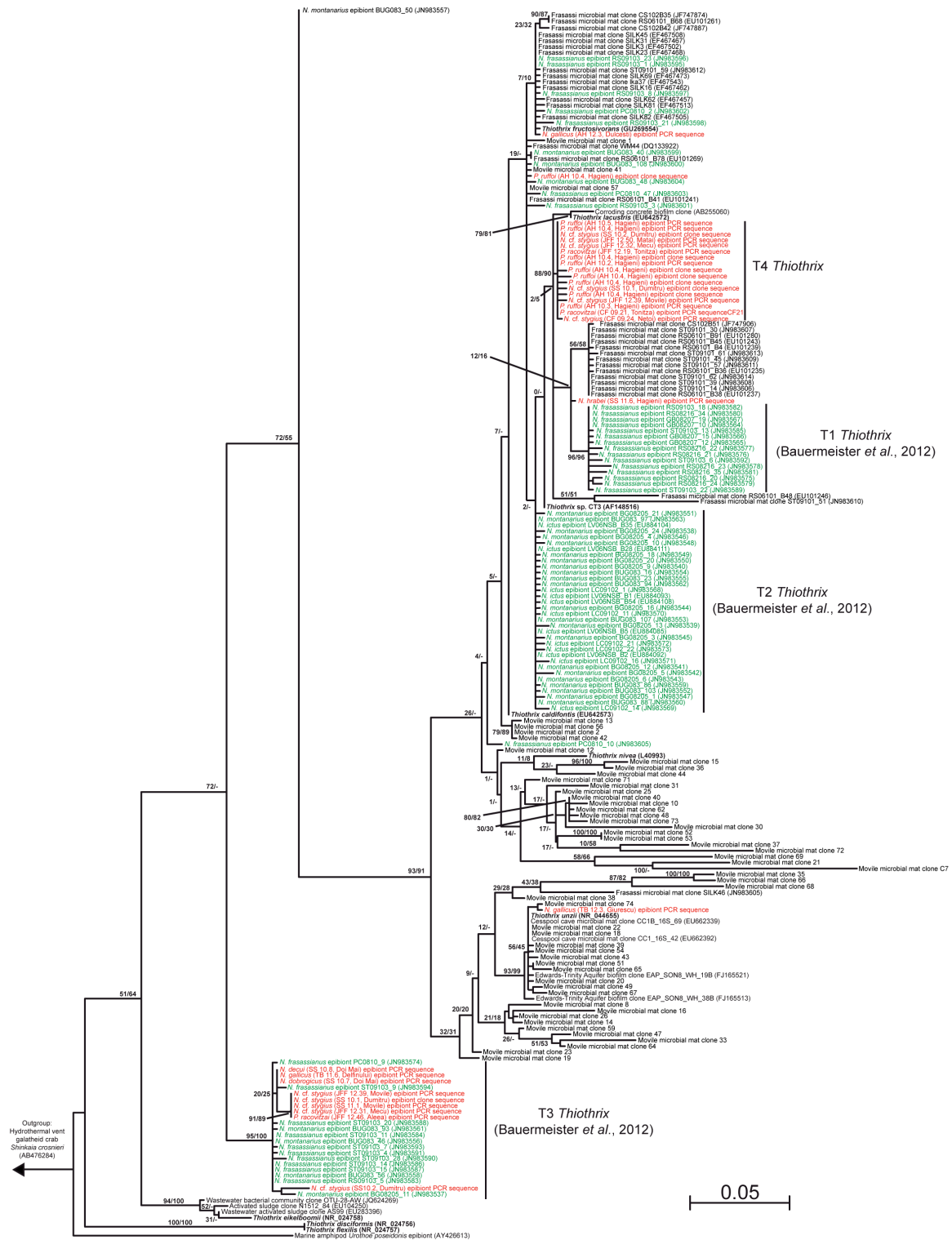


Figure 4.6. Partial 16S rDNA maximum-likelihood phylogenetic tree of cultivated and uncultivated *Thiothrix* species. Cultivated *Thiothrix* strains are in bold. GenBank accession numbers are given in parentheses. Maximum Likelihood/Neighbor-Joining bootstrap values are listed next to the respective nodes. *Thiothrix* epibiont sequences obtained from Romanian niphargid specimens are highlighted in red, those obtained previously from Frasassi-dwelling *Niphargus* species are in green.

Table 4.3. List of localities where niphargid samples for this study were collected.

Town	Location	Date	pH	Temperature (°C)	Conductivity (µS/cm)	H ₂ S (mg/L)	Niphargid species
Hagieni		September 2012		18.1	1080		<i>N. hrabei</i> , <i>P. ruffoi</i>
Mangalia	Mobile Cave	September 2011	7.4	21.2	1071	8.32	<i>N. cf. stygius</i> , <i>P. racovitzai</i>
Mangalia	str. Gheorge Netoi #1A	September 2011	7.4	18.6	1078	4.51	<i>N. cf. stygius</i>
Mangalia	str. Matei Basarab #62	September 2012		19.8	1380		<i>N. cf. stygius</i> , <i>N. decui</i>
Mangalia	str. Matei Basarab #74	September 2012		18.1	1460		<i>N. decui</i>
Mangalia	str. Dumitru Ana #13	September 2011	7.3	19.1	1052	3.42	<i>N. cf. stygius</i>
Mangalia	str. Ion Mecu #51	September 2011	7.7	19.9	1135	2.24	<i>N. cf. stygius</i>
Mangalia	str. Horia Cloşca Crişan #13	September 2012		19.7	1450		<i>N. decui</i>
Mangalia	Aleea Cetăţii #1						<i>N. cf. stygius</i> , <i>N. racovitzai</i>
Mangalia	str. Pictor Tonitza #1	September 2011	7.3	19.0	1242	0	<i>N. cf. stygius</i> , <i>N. decui</i> , <i>P. racovitzai</i>
Mangalia	str. Mihai Viteazu	September 2011	7.6	17.4	1193	0	<i>N. decui</i>
Mangalia	str. Vasile Pârvan #16	September 2012		17.7	3640		<i>N. gallicus</i>
Mangalia	str. Delfinului 10						<i>N. gallicus</i>
Mangalia	str. Delfinului 16	September 2011	7.4	19.1	1473	0	<i>N. gallicus</i>
Mangalia	str. Maior Giurescu 22	September 2012		18.4	2440		<i>N. gallicus</i>
Dulcesti		September 2011	7.6	15.4	1216	0	<i>N. gallicus</i> , <i>N. decui</i>
Vama Veche							<i>N. dobrogicus</i> , <i>N. decui</i>
Albesti		September 2011	7.5	13.8	1445	0	<i>N. decui</i>
Doi Mai	str. Mihail Kogălniceanu 393	September 2011	7.4	16.1	2235	0	<i>N. dobrogicus</i> , <i>N. decui</i>
Limanu		September 2011	7.6	16.2	1092	0	<i>N. dobrogicus</i> , <i>N. decui</i>
Shabla	Magazin Alim						<i>N. decui</i>

4.5 Discussion

A molecular glance at the biodiversity of niphargids in Dobrogea

The combination of molecular markers used in the present study proved sufficient to resolve with high level of confidence the seven niphargid species found in Dobrogea and the placement of these species within a broad phylogeny of niphargid amphipods. ITS was variable enough to distinguish among niphargid species, but too variable to infer the evolutionary relationships among ancient niphargid lineages. 28S could be used to reconstruct a rooted phylogeny of *Niphargus*, but at the expense of resolution at the branch tips. Only COI seems to have the potential to resolve both deep and shallow relationships in the niphargid phylogeny, since it is very variable and at the same time can be easily aligned with outgroup sequences. However, the amount of niphargid sequences available for this marker is currently very scarce.

Contrary to previous hypotheses (Dancău, 1970; Karaman, 1989; Karaman and Sarbu, 1993), both species of *Pontoniphargus* turn out to be nested within one

particularly variable *Niphargus* clade, which suggests that the morphological traits responsible for the positioning of *Pontoniphargus* as a separate genus are not taxonomically significant. Indeed, the main morphological character used to distinguish *Pontoniphargus* from *Niphargus* is the former's flattened uropod III with elongated endopodite: such uropod III, common in other amphipod genera but typically absent in *Niphargus*, was also reported from one individual of *N. hebereri* (Schellenberg, 1937) and appears therefore variable even intraspecifically.

Romanian and Italian sulfide-dwelling amphipods are not closely related but harbor closely related sulfide-oxidizing ectosymbionts

Except for a weakly supported relationship between *N. cf. stygius* from Movile and *N. montanarius* from Frasassi, the niphargids from Dobrogea do not appear to be particularly related with the ones from Frasassi. In this regard, it is quite striking that they harbor identical (T3) or closely related (T4 vs. T1 and T2) sulfide-oxidizing ectosymbionts. As T3 has been found associated with niphargids from both sulfidic and non-sulfidic environments, it is possible that this *Thiothrix* lineage is widely distributed throughout Europe. In contrast, T1 and T4 *Thiothrix* symbionts have so far only been found in sulfidic environments, suggesting that they have independently established symbiosis with niphargids.

4.6 Acknowledgements

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Summary and Conclusion

This thesis project dealt with the exploration of ectosymbiotic relationships between filamentous, sulfur-oxidizing *Thiothrix* bacteria and groundwater-inhabiting niphargid amphipods. In 2009, "the first known example of a non-marine chemoautotroph-animal symbiosis", involving *Niphargus ictus* amphipods and a single *Thiothrix* phylotype, was reported from sulfidic waters of the Frasassi caves in central Italy (Dattagupta *et al.*, 2009). After the subsequent discovery of two more Frasassi-dwelling *Niphargus* species (Flot *et al.*, 2010a), named *Niphargus frasassianus* and *Niphargus montanarius* (Karaman *et al.*, 2010), the question arose whether the *Niphargus-Thiothrix* association is more diverse than previously thought.

Indeed, we discovered that all three Frasassi-dwelling *Niphargus* species harbor *Thiothrix* ectosymbionts, which are predominantly attached to the hosts' legs and antennae (Bauermeister *et al.*, 2012). The ectosymbionts belong to three distinct phylogenetic clades, which we named T1, T2, and T3, and their relative distribution among the *Niphargus* is strongly host species-specific. While T1 occurs exclusively on *N. frasassianus*, T2 is shared between *N. ictus* and *N. montanarius*, and T3 can be present on all three *Niphargus* species. Free-living counterparts of T1–T3 were not found in Frasassi microbial mats, suggesting that the ectosymbionts are transmitted among their hosts via intra- and interspecific inoculations. Symbioses with T1 and T2 have presumably been established after the three *Niphargus* species independently invaded the Frasassi caves. In contrast, T3 appeared to be a more ancient ectosymbiont that was introduced to Frasassi by one or more *Niphargus* species.

Thiothrix bacteria are known to be metabolically versatile (Odintsova *et al.*, 1993; Howarth *et al.*, 1999; Chernousova *et al.*, 2009). We were curious whether the three ectosymbionts have different metabolic capabilities and whether they derive metabolic benefits from the associations with their distinctly behaving *Niphargus* hosts (Flot *et al.*, 2010a). We incubated the three *Niphargus* species and *Thiothrix* mats from Frasassi with ^{13}C -labeled carbon substrates and ^{15}N nitrogen gas and detected carbon and nitrogen incorporation into *Thiothrix* cells with Nano-scale Secondary Ion Mass Spectrometry (NanoSIMS). The analyses revealed distinct metabolic characteristics of the *Thiothrix* ectosymbionts. T1 *Thiothrix* are obligate

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heterotrophs, whereas T3 are facultatively mixotrophic. Metabolic capabilities of T2 *Thiothrix* varied on their two different host species; while being heterotrophic on *N. montanarius*, T2 showed particularly high autotrophic activity as ectosymbionts of *N. ictus*. Moreover, our NanoSIMS results indicated that T2 symbionts of *N. ictus* derive a substantial ecological benefit from 'hitchhiking' on their host. Water currents created by the swimming legs of *N. ictus* possibly supply T2 with enough oxygen even in stagnant and highly sulfidic Frasassi cave water bodies, which are uninhabitable for free-living *Thiothrix*. Besides their ability to utilize both organic and inorganic carbon substrates, T3 ectosymbionts are also capable of nitrogen fixation. We suggested that their high metabolic versatility may have enabled T3 to successfully establish symbioses with all three Frasassi-dwelling *Niphargus* species, which expose them to very different environmental conditions.

Sulfide is toxic to most aerobic organisms (Bagarinao, 1992), and sulfide tolerances of amphipods are generally low (Theede *et al.*, 1969; Knezovich *et al.*, 1996; Sandberg-Kilpi *et al.*, 1999). Nevertheless, *N. ictus* and *N. frasassianus* prosper in sulfidic Frasassi cave waters. A sulfide-detoxifying function of the sulfur-oxidizing *Thiothrix* ectosymbionts for *Niphargus* has been considered by Dattagupta *et al.* (2009), since the bacterial filaments are mainly attached close to the amphipod gills. To test this possibility, we treated individuals of *N. ictus* and *N. frasassianus* with antibiotics to kill their *Thiothrix* ectosymbionts and exposed these animals together with non-treated ones to Frasassi cave water with gradually increasing sulfide concentrations. All individuals, with and without ectosymbionts, survived exposure to sulfide concentrations far higher than those measured in Frasassi, indicating that *N. ictus* and *N. frasassianus* do not rely on *Thiothrix* bacteria to withstand sulfide in their natural habitat. Instead, the amphipods might employ own sulfide detoxification mechanisms. The ecological role of their ectosymbionts remains thus unknown.

Niphargid amphipods are widespread in European groundwater systems (Sket, 1999), which makes it possible that *Niphargus-Thiothrix* ectosymbioses are not unique to the Frasassi caves. To find out whether similar associations also occur in other subterranean freshwater environments, we examined *Niphargus* and *Pontoniphargus* species from the chemoautotrophic Movile cave (Sarbu *et al.*, 1996) and the surrounding Dobrogea region in Romania. Using SEM and *Thiothrix*-specific

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PCR screenings, we found *Thiothrix* belonging to two distinct phylogenetic clades attached to the appendages of numerous amphipods. One of the clades, named T4, exclusively comprised ectosymbionts of Romanian niphargids from sulfidic habitats. The other clade was identical with T3 ectosymbionts from Frasassi and contained *Thiothrix* sequences obtained from host species inhabiting sulfidic as well as non-sulfidic environments. Although niphargids from Frasassi and Dobrogea are phylogenetically not closely related, they appear to share very similar or even identical *Thiothrix* ectosymbionts.

The results of this thesis suggest that ectosymbiotic associations between sulfur-oxidizing bacteria and niphargid amphipods are not rare in European subterranean ecosystems. Especially the wide dispersiveness of T3 *Thiothrix* ectosymbionts deserves attention. The *Niphargus-Thiothrix* ectosymbioses from Frasassi are probably only the tip of the iceberg, and closer examinations of other groundwater- and cave-dwelling invertebrates may reveal that freshwater chemosynthetic symbioses are not limited to gammaridean amphipods. Blind prawns of the species *Typhlocaris ayyaloni* (Caridea, Decapoda), living in the sulfidic water pool of the Ayyalon cave in Israel (Por, 2007), have already been found covered by a conspicuous, “yellowish (bacterial?) film” (Tsurnamal, 2008).

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