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„Morphology and Fitness of the thiotrophic Symbiont *Cand.* Thiobios zoothamnicoli *in situ* and *in vivo*“

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Judith Drexel

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Betreut von:	Univ.-Prof. Dr. Monika Bright

ABSTRACT

Before the discovery in 1981 of *Riftia pachyptila* living on deep sea hydrothermal vents, and containing a chemolithoautortrophic sulphur-oxidising symbiont, it was thought that only photoautotrophic and heterotrophic bacterial symbionts exist. This was the first description of a chemolithoautotrophic symbiont. After this discovery, there was an intense search for other chemolithotrophic symbiosis and this symbiosis is now known to exist in a broad range of habitats and for a broad range of host species. Bacteria capable of chemosynthesis occur in many different lineages, sulfur-oxidising bacteria, however, mainly belong to the *Gammaproteobacteria*. Knowledge on the mechanisms driving the interactions between the host and symbionts involved in chemolithoautotrophic symbiosis is still scarce. The symbiosis between the peritrich, feather-like *Zoothamnium niveum* and its sulphide-oxidising ectosymbiont *Cand.* Thiobios zoothamnicoli constitutes a promising, simple system with which to investigate research questions on functional aspects concerning cooperation and evolution, particularly for chemolithoautotrophic symbiosis. This system reproduces and grows rapidly, has a short lifetime and is the only thiotrophic symbiosis possible to cultivate over several generations.

Until now, one study has been undertaken on the behavior of this symbiosis under different experimental chemical conditions, focused mainly on the fitness of *Z. niveum* exposed to different sulphide concentrations. The morphology and fitness of *Cand.* Thiobios zoothamnicoli was also considered and those ectosymbionts treated under optimal culture conditions were compared to the *in situ* situation whereupon significant differences were noted (Rinke *et al.* 2007).

In the study presented here, *in situ* as well as *in vivo* specimens treated under optimal conditions were also analyzed. Furthermore, the fate of *Cand.* Thiobios zoothamnicoli was investigated under unfavorable conditions either for itself or for it´s host. It is known that the host's diet partly relies on free-living microbes from the surrounding water column (Rinke, 2002). However, it was still to be determined what happens if the host cannot access part of its diet through these free-living microbes. The response of *Cand.* Thiobios zoothamnicoli to a restriction of their host´s diet was also an open research question. Furthermore, the fate of *Cand.* Thiobios zoothamnicoli under a restriction in its usual nourishment - under sulphide starvation

- was still to be investigated. Potential differences arising when this starvation occurs under water-flow or stagnant conditions as well as when *Cand.* Thiobios zoothamnicoli is attached to previously settled swarmers or adult *Z. niveum* colonies were also of interest.

These reearch questions were investigated by treating *Zoothamnium niveum* samples *in vivo* under optimal or suboptimal conditions either for 48 hours or for seven days. The *in situ* samples and those specimens used for *in vivo* treatments were collected in a canal next to a saline in Piran, Slovenia. The specimens treated under optimal *in vivo* conditions (OPNA) were raised from swarmers in a flow-through respirometer aquaria under sulphide supplementation in 32 µm filtered sea water for seven days. In a further approach, specimens were raised from swarmers in flow-through respirometer aquaria for seven days with sulphide supplementation and 0.2 µm filtered sea water (OPA), meaning that under these conditions the host could not feed on free-living microbes from the surrounding water column. To get an insight into what happens when *Cand.* Thiobios zoothamnicoli is kept under sulphide starvation, two approaches were followed: In the first approach, two sets of fully grown colonies with ectosymbionts attached were placed for 48 hours either in a respirometer flow-through aquarium with a flow rate of approximately 50 ml/h of 32 µm filtered sea water and no sulphide supplementation (FLOW) or in a embryo dish filled with 0.2 µm filtered sea water (NO FLOW). In the second approach, colonies were raised from swarmers in flow-through respirometer aquaria with a flow rate of approximately 50 ml/h of 32 µm filtered sea water and no sulphide supplementation for seven days (NONA).

Specimens were subsequently prepared for and scanned with a scanning electron microscope (SEM) and several parameters related to morphology and fitness of the symbionts covering the hosts feeding cells were evaluated and compared with each other and to results from literature. The feeding cells (microzooids) were divided into two parts to discern any differences between the ectosymbionts covering its upper (oral) and lower (aboral) part. The number of cells and the percentage of host surface coverage were determined in an 70 µm² frame positioned at the edge of the oral and the aboral part. The length and width of individual symbionts on both parts of the microzooid were also measured, with up to 70 cells independently examined in a clockwise helical pattern. From this data the volume and cell elongation index were calculated for the individual measured symbionts. Furthermore, the frequency of dividing cells (FDC) as a measure of fitness was measured in the same manner as cell length

and width. The absolute number of dividing cells / 70 µm² as a measure of cell composition on the host surface was also calculated by combining the FDC with the number of cells measured per 70 µm².

The *in situ* and OPNA samples were compared with each other as well as with similar samples from literature (Rinke *et al.* 2007). From this it could be determined that *Cand.* Thiobios zoothamnicoli shows a response to the actual chemical conditions to which it is exposed. These findings support the classification of *Cand.* Thiobios zoothamnicoli as a pleomorphic cell-type (Bauer-Nebelsick *et al.* 1996a,b; Rinke *et al.* 2006). In addition, *Cand.* Thiobios zoothamnicoli was shown to undergo extreme morphological changes under sulphide starvation and it was supposed that this cell type is capable of forming bulging filaments.

To investigate what happens when the host is restricted in its access to free-living microbes, the samples treated under OPNA and OPA conditions were compared. The host treated under OPA conditions was seen to have half the proliferation rate and therefore half the size of the host treated under OPNA conditions. Although sulphide was available in both treatments and both symbiont populations were seen to have an equal proliferation rate, the smaller hosts were not overgrown by the symbionts. It is suspected that the homeostasis between the two partners of this symbiosis is controlled by the host, as supported by other examples of symbiosis in literature (see Douglas, 2010; Brooks & Richards, 1955; Ruby & Asato, 1993; Whitehead & Douglas, 1993).

When *Cand.* Thiobios zoothamnicoli was attached to fully grown colonies and kept under sulphide starvation in the FLOW and NO FLOW treatment, the symbiosis was still maintained after 48 hours. Although the number of ectosymbionts significantly decreased under both conditions, the remaining ectosymbionts were seen to still undergo division and had an enlarged size. These findings led to the conclusion that *Cand.* Thiobios zoothamnicoli is capable of utilizing nutritional sources other than sulphide.

However, the resulting colonies from the NONA treatment were no longer covered by ectosymbionts. This represented the first time aposymbiotic *Z. niveum* colonies have been established under artificial conditions. The fate of the ectosymbionts under these conditions is still to be investigated.

ZUSAMMENFASSUNG

Im Jahre 1981 wurde *Riftia pachyptila* entdeckt. Ein Vertreter der Pogonophora ohne Magen-Darm-Trakt, der einen chemolithoautotrophen Endosymbionten beherbergt (Cavanaugh, 1981; Feldbeck, 1981; Rau, 1981). Zuvor wusste man nur von phototrophen und heterotrophen Symbionten (see Dubilier *et al.* 2008). Diese Entdeckung führte zur intensiven Suche nach anderen chemolithotrophen Symbiosen, welche folglich bei vielen verschiedenen Arten und in unterschiedlichsten Lebensräumen gefunden wurden (see Cavanaugh *et al.* 2006). Chemolithotrophe Bakterien kommen in vielen Abstammungslinien vor, wobei Sulfid-oxidierende Bakterien hauptsächlich zu den *Gammaproteobacteria* gehören (see Dubilier *et al.* 2008).

Das Wissen über kooperative Interaktionen zwischen Wirt und Symbiont sowie die Evolution von chemolithoautotrophen Symbiosen ist gering und die Symbiose zwischen dem Ciliaten *Zoothamnium niveum* und seinem Sulfid-oxidierenden Ektosymbionten *Cand.* Thiobios zoothamnicoli stellt ein gutes, leicht zu manipulierendes System dar, um Einblicke über Evolution und kooperatives Verhalten von Wirt und Symbiont in einer chemolithoautortrophe Symbiose zu gewinnen. Nicht nur, dass *Zoothamnium niveum* eine schnelle Reproduktion und kurze Lebensdauer hat, es ist auch die einzige thiotrophe Symbiose, die man über mehrere Generationen kultivieren kann (Rinke *et al.* 2007).

Es ist bekannt, dass sich der Wirt *Z. niveum* zumindest teilweise von seinem Ektosymbionten ernährt. Zum einen wird von *Cand.* Thiobios zoothamnicoli unmittelbar nach Fixierung ausgeschiedener Kohlenstoff aufgenommen. Zum anderen wird der Ektosymbiont direkt gefressen (Rinke, 2002). Mittlerweile hat sich eine Studie mit der Anpassung – in Bezug auf Veränderung von Morphologie und Fitness - von *Cand.* Thiobios zoothamnicoli an optimale Kultivierungsbedingungen im Vergleich mit *in situ* Proben beschäftigt (Rinke *et al.* 2007), und Unterschiede wurden entdeckt.

In unserer Studie wurde dieser Ansatz wiederholt und weitergeführt. Wir untersuchten selbst *in situ* Proben und solche, die *in vivo* unter optimalen Bedingungen gezüchtet wurden und verglichen sie miteinander, wie auch mit der korrespondierenden Literatur (Rinke *et al.* 2007). Zudem stellten wir uns die Frage, was mit der *Z. niveum* Symbiose passiert, wenn sie *in vivo*

unter ungünstigen Bedingungen – einerseits für *Cand.* Thiobios zoothamnicoli, andererseits für den Wirt – gehalten wird. Was passiert mit *Cand.* Thiobios zoothamnicoli, wenn sein Wirt in seiner normalen Ernährung eingeschränkt wird und keinen Zugang zu freilebenden Mikroben in der Wassersäule hat? Und was passiert, wenn dieses Bakterium unter Ausschluss von Sulfid gehalten wird? Welchen Einfluss hat dabei der Entwicklungszustand des Wirtes? Entwickeln sich *Cand.* Thiobios zoothamnicoli Populationen, die auf einer jungen, erst ans Substrat angedockten Verbreitungseinheit des Wirtes sitzen anders als solche, die mit erwachsenen Kolonien leben? Welchen Unterschied gibt es, wenn Sulfid bei stehender oder fließender Wassersäule ausgeschlossen wird? Um Einblick auf diese Fragen zu bekommen wurden Z. niveum Proben entweder ohne Sulfid oder unter axenischen Bedingungen gehalten.

Alle Proben, die für diese Arbeit untersucht wurden, stammen von unter Wasser liegendem Holz im Sv. Jernej Kanal neben der Saline Sečovlje in Piran, Slovenien. *In situ* Proben wurden unmittelbar für das Rasterelektronenmikroskop fixiert. Andere Proben wurden entweder als ausgewachsene Kolonien für 48 Stunden oder als Schwärmer für sieben Tage unterschiedlichen Bedingungen *in vivo* ausgesetzt. Die ausgewachsenen Wirts-Kolonien mit *Cand.* Thiobios zoothamnicoli wurden für 48 Stunden zum einen in einem `Flow-through respirometer`- Aquarium mit ~ 50 ml/h Fließgeschwindigkeit von 32 µm gefiltertem Wasser (FLOW) und zum anderen in mehreren Embryo Dish mit 0.2 µm gefiltertem Wasser gehalten (NO FLOW). Zum anderen wurden für drei verschiedene Experimente Schwärmer von *Z. niveum* in `Flow-through respirometer`- Aquarien inokuliert, welche darin in sieben Tagen zu Kolonien heranwuchsen. Folgende Zuchtbedingungen wurden hergestellt: Bei einer Fließgeschwindigkeit von ~ 50 ml/h wurde entweder 32 µm gefiltertes Wasser und Sulfid bereitgestellt (OPNA). Mit diesem Experiment wurden die optimalen *in vivo* Proben gezüchtet. Weiters wurde 0.2 µm gefiltertes Wasser und Sulfid bereitgestellt (OPA). *Z. niveum* wuchs dabei unter axenischen Bedingungen auf. Und weiters wurde 32 µm gefiltertes Wasser und kein Sulfid bereitgestellt (NONA), womit *Cand.* Thiobios zoothamnicoli der Zugang zu Sulfid verwehrt wurde.

Die erhaltenen Proben wurden ebenfalls für das Rasterelektronenmikroskop fixiert und mitsamt den *in situ* Proben für das Rasterelektronenmikroskop präpariert. Pro Versuchsansatz wurden von drei *Z. niveum* Kolonien jeweils 15 Fresszellen gescannt, welche folglich für die Erhebung von Parametern in Bezug auf Morphologie und Fitness in einen oberen (oralen)

und einen unteren (aboralen) Teil geteilt wurden. Folgende Parameter wurden erhoben: Die Anzahl von Ektosymbionten sowie deren Deckungsgrad an Wirtsoberfläche wurden innerhalb eines 70 µm² Rahmens – welcher am Ende des oralen und aboralen Teils positioniert wurde – erhoben. Es wurde die Länge und Breite von bis zu 70 Ektosymbionten pro aboralem und oralem Teil der Fresszelle erhoben, wobei spiralförmig im Uhrzeigersinn zufällig gewählte Zellen vermessen wurden. Aus Länge und Breite wurde das Volumen (nach van Veen & Paul, 1979), wie auch der Cell elongation index (CI) berechnet. In gleicher Weise wie Länge und Breite wurde auch die ´frequency of dividing cells` (FDC) – der Prozentsatz an Zellen, die sich in Teilung befinden – erhoben, ein Maß für die Fitness der Ektosymbionten (Hagström *et al.* 1979). Diese Daten wurden in Zusammenhang mit der Anzahl an Zellen pro 70 µm² gebracht, um die absolute Anzahl an teilenden Zellen pro 100 µm² zu berechnen.

Beim Vergleich unserer *in situ* und OPNA Proben miteinander und mit der korrespondierenden Literatur (Rinke et al. 2007), zeigten die *Cand.* Thiobios zoothamnicoli Populationen gleiche Trends von Morphologie und Teilungsrate zwischen oralem und aboralem Teil der Microzooide. So waren Länge, Breite und folglich Volumen der *Cand.* Thiobios zoothamnicoli Zellen am oralen Teil der Microzooide stets signifikant höher als am aboralen Teil, während CI und FDC stets den gegensätzlichen Trend zeigten. Unterschiede zwischen den einzelnen Probensets bestanden in den aktuellen Mittelwerten der erhobenen Parameter. Die gleichen beobachteten Trends unterstützen die Annahme, dass *Cand.* Thiobios zoothamnicoli Zellen am oralen Teil der Microzooide durch das Schlagen der Cilien des Wirtes anderen chemischen Bedingungen ausgesetzt sind als solche Zellen, die den Rest der Wirtsoberfläche besetzen (Rinke *et al.* 2007). Die Unterschiede von genauer Größe und Teilungsrate zwischen den einzelnen Ansätzen wurden als Reaktion auf die gesamten chemischen Bedingungen interpretiert. Diese Ergebnisse bestärken die Bezeichnung von *Cand*. Thiobios zoothamnicoli als pleomorphen Zelltypen (Bauer-Nebelsick *et al.* 1996a,b; Rinke *et al.* 2006). Mikrozooide von *Z. niveum* Proben, welche unter FLOW Bedingungen gehalten wurden, waren teilweise von Filamenten mit Ausbuchtungen besetzt. Es wird angenommen, dass es sich bei diesen Filamenten um *Cand.* Thiobios zoothamnicoli handelt. Natürlich muss diese Annahme auf Basis einer Genanalyse überprüft werden. Wie auch immer, wenn es sich um *Cand.* Thiobios zoothamnicoli handelt, würde diese Entdeckung zu einer Erweiterung der bekannten Zellformen führen, welche *Cand.* Thiobios zoothamnicoli einnehmen kann. Die Funktion dieser Aus-

buchtungen ist unbekannt und es stellt sich die Frage, ob es sich dabei um mögliche Dispersionseinheiten handelt, welche *Cand.* Thiobios zoothamnicoli unter unwirtlichen Bedingungen bildet. Es wäre also wichtig zu wissen, ob sich DNA in diesen Ausbuchtungen befindet und das Einfärben von Proben mit DAPI oder SYBR green wäre eine Überprüfungsmöglichkeit.

Es ist bekannt, dass *Z. niveum* sich zum Teil von *Cand.* Thiobios zoothamnicoli ernährt: zum einen von Kohlenhydrate, welche von *Cand.* Thiobios zoothamnicoli kurz nach der Fixierung ausgeschieden werden, zum anderen wird *Cand.* Thiobios zoothamnicoli direkt gefressen (Rinke, 2002). Um einen Einblick zu bekommen, was mit der Symbiose passiert, wenn *Z. niveum* keinen Zugriff zu seiner anderen Nahrungsquelle – den frei-lebenden Mikroben in der Wassersäule – hat, wurden die Experimente OPNA und OPA verglichen. *Z. niveum* zeigte eine um etwa 50% geringere Teilungsrate unter axenischen Bedingungen. *Cand.* Thiobios zoothamnicoli zeigte hingegen bei beiden Experimenten eine gleiche Anordnung auf der Wirtsoberfläche wie eine gleiche Teilungsrate und trotzdem wurden die kleineren Wirts-Kolonien des axenischen Experiments nicht überwuchert. Im Gegensatz dazu wurde beobachtet, dass tote *Z. niveum* Kolonien von ihren Symbionten überwuchert werden können (pers. obs.). Demnach wird angenommen, dass ein Kontrollmechanismus wirken muss, welcher das Gleichgewicht zwischen Größe des Wirtes und Größe der Ektosymbiontenpopulation aufrecht erhält. Es wird angenommen dass dieser Kontrollmechanismus vom Wirt stammt, wie es auch bei anderen Experimenten in der Literatur der Fall ist (see Douglas, 2010; Brooks & Richards, 1955; Ruby and Asato, 1993; Whitehead & Douglas, 1993).

Neben der Untersuchung, was mit der Symbiose passiert, wenn der Wirt ungünstige Bedingungen vorfindet, wurde auch ein Augenmerk auf mögliche Veränderungen der Symbiose durch ungünstige Bedingungen für den Ektosymbionten untersucht. Dafür wurden die Proben des FLOW und NO FLOW Experiments untersucht. Diese Proben bestanden aus ausgewachsenen Kolonien, welche vor dem Beginn des Experimentes von einer etablierten Ektosymbiontenpopulation bedeckt waren. Auch nach den 48 Stunden Experimentierzeit waren die Wirts-Kolonien noch mit Ektosymbionten bedeckt. Ein Experiment mit gleichen Zuchtbedingungen, aber längerer Laufzeit, zeigte, dass Ektosymbionten noch auf der Wirtskolonie sitzen, wenn diese gestorben ist (pers. obs. Bright, M., 2013). Die Ektosymbionten waren zwar stark in ihrer Anzahl dezimiert, die verbleibenden Zellen zeigten jedoch eine signifikante Volumenzunahme, wie auch immer noch Teilungsaktivität. Keine nennenswerten Unter-

schiede waren dabei zwischen dem FLOW und dem NO FLOW Experiment zu erkennen. Diese Ergebnisse führten zur Annahme, dass *Cand.* Thiobios zoothamnicoli fähig ist, andere Ernährungsressourcen zu verwenden als Sulfid. Es wird angenommen, dass *Cand.* Endoriftia persephone fähig ist von Chemolithoautotrophie zu einer heterotrophen Lebensweise zu wechseln (Robidart *et al.* 2008). Wir nehmen an, dass auch *Cand.* Thiobios zoothamnicoli diese Fähigkeit besitzt, nicht zuletzt aus dem Wissen, dass alle autotrophen Organismen auch gewisse organische Substrate aufnehmen können und wohl Mixotrophie die Norm in der Natur ist (see Karl, 1995). Doch natürlich müssen metagenomische und funktionelle Untersuchungen unternommen werden, um herauszufinden, ob *Cand.* Thiobios zoothamnicoli einer heterotrophen Lebensweise befähigt ist.

Im Kontrast zu den Ergebnissen des FLOW und NO FLOW Experiments waren die erhaltenen Kolonien vom NONA Versuch aposymbiotisch. Dieses Ergebnis zeigt zum ersten Mal, dass es möglich ist, eine aposymbiotische Z. niveum Kolonie unter *in vivo* Bedingungen zu erhalten. Es wird angenommen, dass diese Kolonien keine Ektosymbionten mehr besitzen, weil im Gegensatz zu den bereits im Vorfeld ausgewachsenen Kolonien des FLOW und NO FLOW Experiments diese Kolonien unter ungünstigen Bedingungen heranwuchsen. Dabei könnte die Ektosymbiontenpopulation nicht nur durch folgende Faktoren dezimiert worden sein, welche auch für das FLOW und NO FLOW Experiment in Frage kommen: höhere Sterberate unter ungünstigen Bedingungen, das Verlassen des Wirtes oder das Abgeschiedenwerden vom Wirt. Beim heranwachsenden Wirten im NONA geschieht wahrscheinlich auch eine Verdünnung der Population, hervorgerufen durch die Zunahme an Wirtsoberfläche durch dessen Wachstum. Wie auch immer, durch welchen Mechanismus eine *Cand.* Thiobios zoothamnicoli Ektosymbiontenpopulation unter Ausschluss von Sulfid entweder dezimiert oder ganz ausgelöscht wird, ist in Frage gestellt. Weiters ist es von Interesse, ob eine aposymbiotische Kolonie fähig ist, ihren Lebenszyklus zu vollführen und ob aposymbiotische Kolonien wieder mit einem Ektosymbionten reinfiziert werden können. Man weiß nichts von einer freilebenden *Cand.* Thiobios zoothamnicoli Population. Wie wir sehen können, stehen noch viele Fragen in Bezug auf diese Symbiose offen. Die Tatsache aber, dass es möglich ist aposymbiotische Kolonien unter artifiziellen Bedingungen zu erzeugen, erweitert die Möglichkeiten an Experimenten, die durchgeführt werden können, um Antworten zu bekommen.

TABLE OF CONTENT

1 INTRODUCTION

1.1 Symbiosis and mutualism

More than half of all species in the world live in or on the body of another species (see Townsend *et al.* 2003). Besides antagonistic interspecific interactions such as parasitism, organisms form alliances in favour of better defence against biotic hazard such as predation or competition and for better prerequisites to handle unfavourable abiotic conditions such as low nutrient availability. Several classifications and definitions are used in literature to describe long-term interactions between two distinct species (see Douglas, 2010). The primary description of the term symbiosis originated from Anton de Bary (1879) as **any type of living together of two distinct species over a longer time period, including parasitism, commensalism, and mutualism**. Many authors argue that the inclusion of all types of long-term interspecies interactions, also embracing unfavourable ones, it is impractical in determining the main features of symbiosis (see Douglas, 2010). However, the term symbiosis is `usually restricted to interactions in which both species benefit´ (Oxford Dictionary of Biology 6 th Edition, 2008), which may be defined as **`symbiosis as a persistent mutualism´** (see Douglas, 2010). The term mutualism describes `an interaction between two species in which both species benefit´ (Oxford Dictionary of Biology 6 th Edition, 2008), whereupon both members of the association receive an increase in fitness as each partner receives benefits from its counterpart which are greater than its own costs to provide the benefit to the other. (see Douglas, 2010). In this paper, the second definition will be used.

A mutualistic relationship can result in access to new metabolic capabilities, as known for the endosymbiosis theory for eukaryotic cells. Their ancestors lacked the abilities of aerobic respiration and photosynthesis and gained access to these processes through the uptake of microorganisms, which became symbiotic rather than being digested. Another interesting example is the ability of a wide range of biota to live in dark, deep-sea environments by gaining the energy they require through symbiotic relationships with chemolithotrophic microorganisms (see Douglas, 2010).

1.2 Chemolithotrophic symbioses

Chemolithotrophy is the ability to obtain metabolic energy through the oxidation of inorganic compounds such as iron, nitrogen and sulphur. Chemolithoautotrophs use this energy to synthesize organic compounds from an inorganic carbon source such as CO_2 (see Karl, 1995).

1.2.1 Research history

Prior to the discovery of *Riftia pachyptila* only phototrophic and heterotrophic symbiosis were known (see Dubilier *et al.* 2008). In 1977, the submersible *Alvin* discovered *R. pachyptila* at the hydrothermal vents along the Galapagos Rift. It was originally hypothesized that these worms receive their nutrition through filter-feeding on either organic compounds translocated from higher water masses through hydrothermal convection or chemoautotrophic bacteria living on the vent side (Lonsdale, 1977). However, in 1981, it was shown that gram-negative bacteria and active enzymes involved in sulphide-oxidation and carbon fixation were present within the trophosome – a highly vascularized organ within the worms trunk – and that *R. pachyptila* receives chemoautotrophically produced carbohydrates (Cavanaugh *et al.* 1981; Feldbeck, 1981; Rau, 1981). These findings led to the conclusion that *R. pachyptila* harbours a chemosynthetic endosymbiont, resulting in the discovery of chemosynthetic endo- or ectosymbionts for most vent taxa as well as the research of similar symbiosis in other habitats (see Cavanaugh *et al.* 2006).

1.2.2 Sulphur-oxidizing symbionts

Chemolithoautotrophic sulphur-oxidizing symbionts all belong to the Proteobacteria, within the classes *Alpha*-, *Gamma*- and *Epsilonproteobacteria* (see Ott *et al.* 2004). Most belong to the *Gammaproteobacteria*, partitioned into at least nine different clades. Most clades also contain free-living members, which leads to the assumption that sulphur-oxidizing symbiosis evolved several times independently (see Dubilier *et al.* 2008).

They can occur as endo- or ectosymbionts. Endosymbionts live either intracellularly in special organs such as in the trophosome of vestimentiferan tubeworms (Cavanaugh, 1981) or extra-

cellularly in organs of other functions as in the intestinal lumen of the nematode *Astromonema southwardorum* (see Giere, 1996). However, many appear as ectosymbionts - living on the body surface of their eukaryotic host - as is the case for Nematoda (Stilbonematinae), particular Annelida (Alvinellidae), arthropods and ciliates (see Ott *et al.* 2004).

So far it has not been possible to cultivate chemoautotrophic or methanotrophic symbionts in pure culture, therefore the detection of the enzymes required for chemosynthesis and inorganic carbon fixation is the only way to detect their metabolic character (see Cavanaugh *et al.* 2006).

1.2.2.1 Carbon fixation

Different pathways through which microorganisms reduce CO_2 to organic matter are known today, whereby the use of more than one pathway simultaneously has been proven for several microorganisms (see Colby *et al.* 1979).

Besides the Calvin-Benson-Bassham cycle – which has two key enzymes, the D-ribulose-5 phosphate kinase and the RuBis-CO – examples of alternative autotrophic methods of CO_2 fixation and reduction are the ribulose monophosphate (RuMP) pathway, the serine pathway, the carbamyl phosphate synthetase pathway, and two distinct acetyl CoA (coenzyme A) pathways: the cyclic reductive TCA cycle and the noncyclic reductive acetyl CoA pathway (see Karl, 1995). Additionally, microorganisms are capable of catalysing the anaplerotic fixation of CO_2 to restock the necessary amount of biosynthetic intermediates such as malate, oxalacetate and succinate (see Quayle & Ferenci, 1978; Wood, 1989).

These alternative autotrophic pathways may play a major role than the Calvin-Benson-Bassham cycle, at least in oxygen limited habitats such as on deep-sea hydrothermal vent sides (see Karl, 1995). For example, proteomic analysis of the *Riftia pachyptila* endosymbionts led to the assumption that this microorganism is using the reductive TCA cycle to a greater extent than the Calvin-Benson-Bassham cycle to incorporate inorganic carbon. The lower amount of energy required to synthesize triose phosphate by the TCA pathway as compared to the Calvin-Benson-Bassham cycle may be the determining factor behind this assumption (Markert *et al.* 2007). While for the reductive TCA cycle only 4 to 5 mol ATP are required for triose phosphate synthesis, 9 mol of ATP are necessary for the Calvin-Ben-

son-Bassham cycle (Fuchs, 1989).

However, it is assumed that chemolithoautotrohic microorganisms are rather mixotrophic than obligate autotrophic. It is known that all autotrophic microorganisms are also able to use organic compounds such as acetate as carbon sources (see Karl, 1995). For example, the *Riftia pachyptila* symbiont was shown to be able to store glycogen, which may be used as an energy source under environmentally low sulphide concentrations by heterotrophic pathways - glycolysis and the oxidative TCA cycle (Markert *et al.* 2007).

1.2.2.2 Sulphur oxidation

Sulphur oxidation can occur over different metabolic pathways, although enzymes like AP sulphurylase indicate sulphide oxidation (see Cavanaugh *et al.* 2006).

Besides the - debated - capability of the enzymes sulphide:quinone oxidoreductase (Sqr) and flavocytochrome *c* (Fcc) to oxidise sulphide to elemental sulphur and the enzyme sulphite oxidase (SO) to catalyse the oxidation of sulphide to sulphate, two main pathways of sulphur-oxidation are known to exist in *Gammaproteobacteria:* (1) the Sox multienzyme system and (2) the reverse sulphate reduction (Yamamoto & Takai, 2011).

The **Sox multienzyme complex** system was found in *Paracoccus* sp., whereby sulphur-compounds are bound to the SoxY protein and protons are gained through the activity of thiosulphate-dehydrogenase and persulphate-dehydrogenase: The intermediate sulphate is released through hydrolysation by the enzyme sulphatase. Energy is not gained through the sulphide-oxidation but through the protons passing through the respiration chain. The **reverse sulphate reduction** pathway occurs in *Acidianus ambivalens*, whereby sulphide is oxidised to sulphate to generate ATP through a bidirectional substrate-level phosphorylation with the enzyme adenosinephosphosulphate-(APS-)reductase and a APS-phosphate-adenyltransferase. Another way to build ATP in *Acidianus ambivalens* is through a proton-gradient. The sulphur-oxygenase/reductase enzyme, located in the cytoplasm, disproportionates sulphur to sulphide and sulphate through the use of oxygen. Either the sulphide is converted to thiosulphate, which is oxidised to tetrathionate by the thiosulphate-chinone-oxidoreductase, or the sulphide is oxidised to sulphate by the enzyme sulphide-chinone-oxidoreductase. Both reactions energise a membrane bound chinone-chinole electron-translocating complex, generat-

ing the proton-gradient (see Fuchs & Schlegel, 2006).

1.2.3 Habitats and hosts harbouring chemolithotrophic symbiosis

Chemosynthesis is found in habitats in which a chemical gradient or a chemocline occurs. This is where inorganic, high energy compounds such as sulphide or methane are separated from the oxygenated water column. Exposed sulphate from marine sediments is mainly reduced to sulphide by microorganisms and therefore available for sulphide-oxidation. In contrast, on hydrothermal vents sulphur contained in rocks and seawater is geothermally reduced to sulphide (see Cavanaugh *et al.* 2006).

The range of habitats in which chemosynthesis occurs is broad, including hot vents and cold seeps, mud volcanoes, whale and wood falls, continental margins and shallow-water coastal sediments. Host species known today include the phyla Annelida, arthropoda, mollusca, nematoda, porifera, platyhelminthes and ciliophora (see Dubilier *et al.* 2008).

1.3 Symbiosis of *Zoothamnium niveum* and *Cand.* Thiobios zoothamnicoli

The symbiosis between *Cand.* Thiobios zoothamnicoli and its host *Zoothamnium niveum* is particularly advantageous to investigate as it has been found to be the only thiotrophic symbiosis possible to cultivate over several generations (Rinke *et al.* 2007). Further advantages are the easy access to this symbiosis living in shallow-water habitats (see Ott *et al.* 2004) and the fast generation time of *Z. niveum,* with an individual colonie's life-span estimated at 7 days (Ott *et al.* 1998).

1.3.1 Occurrence

Zoothamnium niveum have thus far been found in the Red Sea (Hemprich & Ehrenberg 1831; Ehrenberg, 1838), the Gulf of Mexico and the Caribbean Sea on mangrove peat walls (Bauer-Nebelsick *et al.* 1996a) and sunken dead wood (Laurent *et al.* 2009). They have also been found in the Mediterranean Sea on degrading leaf debris of the sea grass *Posidonia oceanica* and *Cystoseira* sp. as well as nearby rocks (see Ott *et al.* 2004) and submerged pieces of dead wood (pers. Obs.). Further locations have been the Atlantic ocean at the coast of Lan-

zarote (Canary Islands) on crevices (see Wirtz & Debelius, 2003) and in Tokyo Bay on a deployed whale vertebra at a depth of 5 m (Kawato *et al.* 2010).

Z. niveum can be found in groups of more than a hundred colonies, strongly aggregated when inspected on a m² scale, as found on mangrove peat walls in the Caribbean Sea (Ott *et al.* 1998). Small patches typically consist either of small young colonies or large senescent ones showing loss of microzooids and strong overgrowth on the basal part of the stalk. Large patches contain colonies of all sizes.

Investigation of dead wood showed that the *Z. niveum* symbiosis is a pioneer colonizer. Colonies appear when sulphide exposure starts to occur and permanently colonize the wood with a high abundance at times of maximum, more stable sulphide concentrations as well as when strong fluctuations occur, with a wide amplitude in sulphide concentrations of between > 1mM and undetectable (Laurent *et al.* 2013).

1.4 Zoothamnium niveum

1.4.1 Ciliates

As of 1991, 8000 species of the phylum Ciliophora were known, which constitutes the group with the most kindred species within the kingdom Protista. All known ciliophorans are heterotrophic of which many are phagotrophic and the rest are mouthless endosymbionts. They are heterokaryotic and at least once upon their lifetime ciliated. Most ciliates are mobile. The phylum is sub-divided into eight different classes, which also cover the main evolutionary lineages. The class Oligohymenophorea delineates from the other classes by possessing only a few adoral membranelles (polykinetids) – typically a total of three. The sub-class Peritrichia, containing genera such as *Vorticella*, *Carchesium* and *Zoothamnium*, consists mostly of sessile and stalked bacterivorous ciliates (see Harrison & Corliss, 1991).

1.4.2 Phylogeny

The host of *Cand.* Thiobios zoothamnicoli was originally described as *Zoocladium niveum* by Hemprich and Ehrenberg in 1831 from one specimen from the Red Sea. It was later classified with the genus Zoothamnium Bory de St. Vincent, 1824 (see Ehrenberg, 1838). *Zoothamnium*

niveum was redescribed by Bauer-Nebelsick based on populations from Belize (1996a,b).

A phylogenetic 18S small subunit ribosomal RNA (ssu rRNA) comparison of seven different *Zoothamnium* species clustered all species except *Z. arbuscula* in one group, of which *Z. niveum*, *Z. alternans* and *Z. pelagicum* composed one clade. The *Z. niveum* samples used for this comparison derived from Pete Stone´s Creek, St. Lucie County, Florida (Clamp & Williams, 2006).

1.4.3 Morphology

The feather-like eukaryotic cell colony consists of a basal adhesive disc and a central stalk with alternating branches, whereby second fans can be established (see FIG.1). The entire colony, except the proximal end of the stalk and the adhesive disc, is able to contract due to spasmoneme. Three cell-types are distinguishable: *Terminal zooids* occur at the distal ends of stalk and branches. These are the only cell-type capable of longitudinal fission (see also below) and are therefore responsible for vegetative growth of the colony from which the other cell-types subsequently emerge (Kloiber *et al.* 2009). *Macrozooids* occur at the proximal end of branches and represent the dispersive unit translocating after constriction from the mother colony with ciliated telotrochal kinetosomes. *Microzooids* are alternately applied on the branches and possess an active complex oral ciliature and a functioning cytopharynx, with the duty of filter-feeding. The entire colony, aside from the most basal part of the stalk and the adhesive disc, is covered in a monolayer of ectosymbionts (Bauer-Nebelsick *et al.* 1996a). While micro- and macrozooids both possess a similar oral ciliature, a fully developed cytopharynx and food vacuoles are only found in microzooids, which commonly contain bacteria similar to the ectosymbionts (Bauer-Nebelsick *et al.* 1996b).

Through BrdU labeled cell nuclei residing in the S-phase, it was found that top terminal zooids had an unlimited cell division rate, terminal zooids had an age dependent proliferation rate decreasing from distal to proximal and macrozooids had an intense DNA synthesis while connected to the mothers colony. In contrast, microzooids did not show any initiations for division (Kloiber *et al.* 2009).

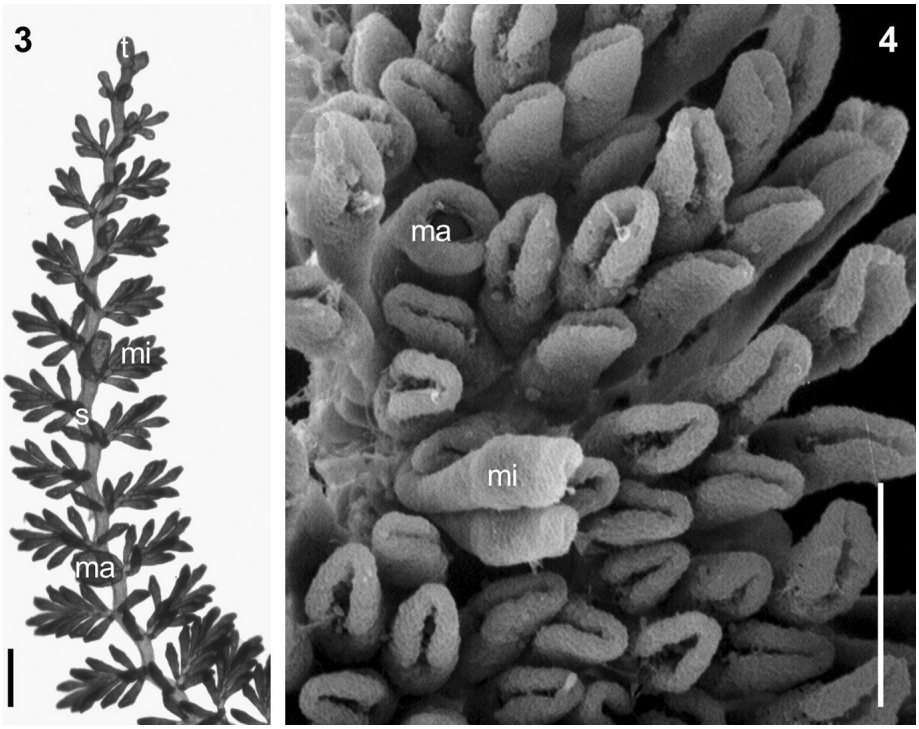


FIG. 1. (3) LM micrograph of *Zoothamnium niveum* colony with stalk (s) and terminal zooid (t) on its tip and alternate branches with microzooids (mi) and macrozooids (ma); scale bar = 100 mm. (**4)** SEM micrograph of contracted colony showing several microzooids (mi) and one macrozooid (ma) covered by symbionts; scale bar = 50 mm. (see Ott *et al.* 2004)

1.4.4 Microbial fouling

Zoothamnium species show tolerance to microbial fouling (Bauer-Nebelsick *et al.* 1996a). Old, senescent *Z. niveum* species have been seen to be overgrown by other microorganisms such as white filamentous bacteria, stalked bacteria and diatoms, initiating at the basal part of the stalk and dispersing to the point where the ectobacterial coat is lost (see Ott *et al.* 2004).

It has been hypothesized that this microbial fouling may have been necessary for the evolution of the symbiotic relationship with *Cand.* Thiobios zoothamnicoli. As a member of the fouling community, the ectosymbionts found favourable conditions by getting access to H_2S and O_2 much more easily through their host's pattern of movement and a relationship was therefore established (see Ott *et al.* 1996).

1.5 *Cand.* Thiobios zoothamnicoli

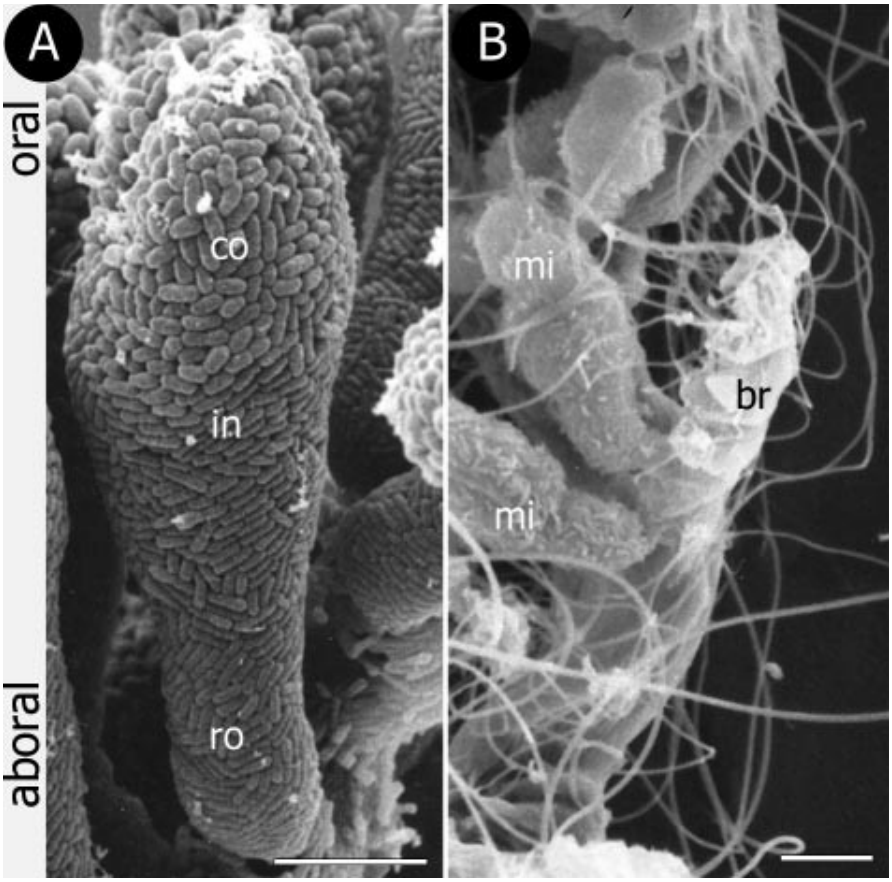
1.5.1 Phylogeny

Electron microscopic studies identified the ectosymbionts of *Z. niveum* as gram-negative bacteria due to their cell wall (Bauer-Nebelsick *et al.* 1996b). By sequencing the 16S rRNA gene and using fluorescence in situ hybridization it could be proven that the ectosymbiotic community consists of one single pleomorphic phylotype of *Gammaproteobacteria,* given the species status `*Cand.* Thiobios zoothamnicoli´. The highest 16S rRNA gene similarity was found for one free-living sulphur oxidizing bacterium ODIII6 and the endosymbiont of a hydrothermal vent gastropod from the Indian Ocean. Together they constituted a monophyletic group within the *Gammaproteobacteria* (Rinke *et al.* 2006).

Sequenced 16S rRNA and intergenic spacer regions of *Cand*. Thiobios zoothamnicoli populations from different locations were compared, whereby a distinction on strain level was detected. A population from mangrove peats at Twin Keys, Belize shows a similarity of 99.7% to a population from seagrass debris at Calvi, Corsica (Rinke *et al.* 2009). Both have a 99.2% 16S rRNA similarity to a population from a deployed whale bone at a depth of 5m in Tokyo Bay. All three populations compose a monophyletic group (Kawato *et al.* 2010).

1.5.2 Morphology

The ectosymbionts of *Zoothamnium niveum* were detected when redescribed by Bauer-Nebelsick *et al.* (1996 a,b). These obligatory symbionts cover the entire surface except the adhesive disk and the most basal part of the central stalk – where matured colonies show an overgrowth with other bacteria - as a monolayer containing two different morphotypes (see FIG. 2). They mainly appear rod-shaped (average length, 1.4 µm; average width, 0.4 µm) in regular, zig-zag order, changing to a more coccoid shape (average length, 1.9 µm; average width, 1.0 µm) at the oral part of microzooids, with a smooth transformation of morphotypes in between. The coccoid-shaped cells show no strict order, sometimes composed as pseudo-multilayer with not every cell contacting the host surface (Bauer-Nebelsick *et al.* 1996a).



. **FIG. 2.** SEM of the *Zoothamnium niveum* symbiosis. **(A)** *Z. niveum* microzooid with bacterial coat, consisting of coccoid rods (co) on the oral part of the microzooid, rods (ro) on the aboral part, and transitional stages of intermediate shapes (in) in between. **(B)** Basal part of the *Z. niveum* colony exhibiting large attached filaments on branch (br). Note that the ectosymbiotic coat on the microzooids is partly lost. Bar lengths, 10 µm. (Rinke *et al.* 2006)

1.5.3 Metabolism

A chemolithoautotrophic, sulphur-oxidizing activity of the ectosymbionts was suspected, with the ectosymbionts receiving their reactants – separated by a chemocline – through translocation facilitated via contraction and expansion by the host (see Ott *et al.* 1996). As previously supposed (Bauer-Nebelsick *et al.* 1996a,b), elemental sulphur inclusions (S_8) occur within the ectosymbionts, as proven by Raman spectrometric analysis in connection with Transmission and Scanning Electron Microscopy (Maurin *et al.* 2010). The detection of the *cbbL* gene, coding for RuBis-CO, indicate autotrophy by using the Calvin Cycle for CO_2 fixation. Furthermore,

it is suspected that the energy necessary for respiration activities and autotrophy derives from the reverse sulphate reduction pathway, by highlighting the genes *apsA*, *apsB* and dsrAB which encode for the large subunits (α and β) of adenosine-5´-phosphosulphate (APS) reductase and the dissimilatory sulphite reductase, respectively (Rinke *et al.* 2009).

1.6 Transmission mode

The mode of transmission has large influence on morphology, physiology and evolution for both the host and its symbiont (see Bright & Bulgheresi, 2010). In *Z. niveum* the symbionts are vertically transmitted (Bauer-Nebelsick *et al.* 1996a). In vertical transmission the symbionts are handed over from the parents to their offspring and for this reason the association is permanent with no aposymbiotic life-phase for the host (see Bright & Bulgheresi, 2010). It has been estimated that about 90,000 symbionts cover a typical swarmer – the dispersive unit of the asexually reproducing *Z. niveum* - with a diameter of 150 µm (Bauer-Nebelsick *et al.* 1996a). When this swarmer settles and start to divide the symbiont's population grows accordingly, covering all new established host surfaces except the adhesive disc and about 300 – 500 µm of the basal stalk (Bauer-Nebelsick *et al.* 1996a).

1.7 Dynamics of the chemical environment in connection with host behaviour

It was previously assumed that the alternating contraction and expansion pattern of *Z. niveum* constitutes the main force through which *Cand.* Thiobios zoothamnicolis accesses both sulphide (H_2S) and oxygen (O_2) by moving through the chemocline from the H_2S rich boundary layer to the oxygenated overlaying water-column. Due to high Reynolds number when contracting, the water does not adhere to the host's surface and is exchanged, whereby no oxygenated seawater is dragged down through the chemocline. The opposite situation is assumed when the colony slowly expands, whereby the sulphide rich water is dragged upwards through the chemocline (Ott *et al.* 1998). These actions were revealed through microelectrode measurements *in vivo* (Vopel *et al.* 2001, 2002; Røy *et al.* 2009) and *in situ* (Vopel *et al.* 2005; Røy *et al.* 2009).

The contraction and expansion pattern of *Z. niveum* causes high velocity in the surroundings

adjacent to the host and mixing of both water bodies (Vopel *et al.* 2001), whereby the contracted colony becomes totally encased by water from above and the expanded feeding colony is surrounded by several current systems (Røy *et al.* 2009).

The adjacent water migrates into a feeding current, which is generated by the cilia of the host's microzooids, whereby the water flows unidirectionally from the convex to the concave side through the colony over its entire length (Røy *et al.* 2009). In connection with the feeding current, a unidirectional, perpendicular water flow streaming downwards at the surface of the colony occurs (Vopel *et al.* 2002). Additionally, the feeding current produces a vortex current system at the tip of the colony, which does not come in contact with the colony itself. A background current system also exists, which consists of water flowing over the peat surface (Røy *et al.* 2009). *In situ* observations of *Z. niveum* patches revealed that the water currents at the substrate surface and the feeding current of the ciliates result in the chemical properties advantageous for *Cand.* Thiobios zoothamnicoli and its host. The host resonates with alternating periods of high and low speed sea water currents within tenths of a second along the substrate surface on which the colonies reside. By orientating itself almost parallel to the substrate surface during high speed sea water currents, the host is embedded in H_2S-rich deposits on the substrate surface. During low speed sea water currents, the host orientates itself perpendicular to the substrate surface and the feeding current it generates replaces the H_2S-rich water adhered to the colony with O_2-rich water. By generating pressure differences the feeding current also facilitates the regeneration of the H_2S rich boundary layer through diffusion out of the substrate (Vopel *et al.* 2005).

Only a slight mixing occurs between the different current systems due to the low Reynolds number and resulting high viscosity of the sea water (Røy *et al.* 2009). However, the water-flow pattern determined for an individual colony cannot be viewed in isolation from those water-flow patterns generated by other colonies in the immediate surroundings, which are likely to have an influence on each others chemical and physical conditions (Vopel *et al.* 2002).

1.8 Trophic relationship

The feeding current generated by the hosts leads to easier access to O_2 for *Cand.* Thiobios zoothamnicoli in favour of faster H_2S-oxidation (Vopel *et al.* 2002). The rate of sulphide uptake by ectosymbionts covering microzooids under the water current situation was estimated to be twice as high as under stagnant conditions (Røy *et al.* 2009).

It is now assumed that the function of host´s contraction and expansion is to clean large particles from its ciliature. As a side effect of the sheer stress generated by contraction, cell-shrinkage and bunching of the zooids, ectosymbionts become detached. The ectosymbionts therefore become associated with the feeding current and ultimately end up in the cytopharynx of a microzooid (Vopel *et al.* 2002). Electron microscopic analysis of host's cytopharynx and food vacuole showed that the bacteria contained within all had the same morphological characteristics as the ectosymbionts (Bauer-Nebelsick *et al.* 1996b). Through ^{14}C bicarbonate pulse/chase experiments it was proven that part of the host´s diet is reliant on its symbionts. The carbohydrates autotrophically built up by the ectosymbionts are thereby gathered in two ways: Low molecular weight carbohydrates exudated by ectosymbionts end up in the feeding current and/or are absorbed through the cell wall. The second method is through the host feeding directly on the ectosymbionts (Rinke, 2002).

1.9 Cultivation

Cand. Thiobios zoothamnicoli and its host *Zoothamnium niveum* have been cultivated over several generations, where optimal growth of the host occurs at a sulphide concentration of 3 – 33 µmol L^{-1}. The use of a flow-through respirometer aquarium allows exposure to constant chemical conditions, in contrast to the fluctuations found in nature. This approach allows *Z. niveum* and its symbiont to be exposed to various chemical conditions at a steady flow - and to simulate stress situations i.e. through sulphide exclusion - and the resultant morphological and physiological changes of both to be followed. Cand. Thiobios zoothamnicoli covering the terminal zooids and the mircrozooids were observed under *in situ* and optimal *in vivo* conditions. The *in situ* samples were sampled in Calvi, Corsica, France grown on 0.02 – 2.7 m^3 debris accumulations of *Posidonia oceanica* and *Cystoseira* spp. found at a depth of between

8.4 and 14.9 m. *In vivo* samples were cultured for 14 days under stable flow conditions in 100 µm filtered sea water and 12 µmol L^{-1} sulphide. The ectosymbiont's morphology was evaluated by measuring their length and width in order to calculate their volume and the cell elongation index (the ratio of length to width). Their fitness was evaluated by measuring the frequency of dividing cells (FDC) by the method of Hagström (1987). It has been found that the ectosymbionts under optimal *in vivo* conditions are much smaller and more rod-shaped, with a higher FDC on all parts of the microzooids compared to the *in situ* ectosymbionts (Rinke *et al.* 2007).

1.10 Research question

As mentioned above, the host´s diet relies partly on carbohydrates produced by its autotrophically active ectosymbiont *Cand.* Thiobios zoothamnicoli (Rinke, 2002). The cultivation of *Z. niveum* and its symbiont is possible and optimal conditions for cultivation could be identified (Rinke *et al.* 2007). Several investigations of the growth rate – an indicator of fitness - of *Z. niveum* under optimal and suboptimal conditions have been undertaken, while the morphology and fitness of *Cand.* Thiobios zoothamnicoli have only been investigated under *in situ* and optimal *in vivo* conditions. But what happens to *Cand.* Thiobios zoothamnicoli under abiotic and biotic stress situations? Is the symbiosis still maintained under stress conditions, especially when the host is starving or when *Cand.* Thiobios zoothamnicoli suffers sulphide preclusion? What happens to *Cand.* Thiobios zoothamnicoli if the host cannot access part of its diet through free-living microbes from the water column? What happens to *Cand.* Thiobios zoothamnicoli under sulphide starvation? Subsequently, what differences arise when *Cand.* Thiobios zoothamnicoli is attached to previously settled swarmers or adult *Z. niveum* colonies? What influence do stagnant or flow conditions have on *Cand.* Thiobios zoothamnicoli? These issues have been investigated by raising swarmers and keeping colonies in flow-through respirometer aquaria or embryo dishes under stress conditions for 48 hours or seven days. Changes in symbiont morphology and fitness are evaluated and samples from corresponding treatments are compared with each other, with the situation *in situ* and/or under optimal conditions *in vivo* and, when available, with results from related treatments in literature.

2 MATERIAL & METHODS

2.1 Maintenance of *Zoothamnium niveum* symbiosis

2.1.1 Collection in the field

Submerged pieces of wood with *Zoothamnium niveum* attached were collected by snorkeling at a depth of between 50 cm and 1.5m in the Sv. Jernej canal (3.2 km long) next to the saline of Sečovlje, Piran, Slovenia in October 2012. The surface water had a temperature of 20-20.7 °C, a pH of 8.12 and salinity of 34.4 ‰. These pieces of wood were transported in buckets of seawater to the laboratory where they were stored in containers with a flow-through seawater supply for up to 23 days (Table 1).

Wood Block #	Collection Date	Start of Experiment:
5	03/10/12	21/10/12; 26/10/12
6	03/10/12	21/10/12; 26/10/12
19	16/10/12	17/10/12; 18/10/12; 19/10/12
20	16/10/12	17/10/12; 18/10/12; 19/10/12
21	16/10/12	17/10/12
22	21/10/12	22/10/12
23	21/10/12	22/10/12
24	21/10/12	22/10/12
25	21/10/12	21/10/12; 22/10/12
26	21/10/12	22/10/12
28	21/10/12	21/10/12; 22/10/12
29	21/10/12	21/10/12; 22/10/12
30	10/21/12	22/10/12

Table 1: Number and collection date of the wood blocks and start date of experiment with colonies isolated from wood blocks.

2.1.2 Isolation from the wood

Aggregations of *Zoothamnium niveum* were cut from the wood pieces with a scalpel and these smaller fragments of wood were collected in a petri dish with 0.2 µm filtered seawater. Individual colonies were isolated under the dissecting microscope by cutting them at the lower part of the stem from the fragments of wood with a MicroPoint™ Scissor. The colonies were cleaned from debris by rinsing them with 0.2 µm filtered seawater twice.

2.2 Flow-through respirometer system

Prior to start of the experiments with the flow-through respirometer system (Rinke *et al.* 2007), the flow-in seawater and sulphide solution used in these experiments were prepared. A disinfected 20 litre canister was filled with either 32 µm (called non-axenic) or 0.2 µm (called axenic) filtered seawater and air bubbled through continuously. Where the experiment was ran with a sulphide supply, either a 2 mM or a 1.5 mM sulphide solution – for use in an aquarium with a volume of 36 ml or 6 ml respectively - was prepared by dilution of dry sodium sulphide in nitrogen-saturated, oxygen free water. This dilution was filled into a 100 ml syringe. The flow-through respirometer system (see FIG. 3) consists of an acrylic glass aquarium with a removable glass plate cover. This chamber is connected to the canister (mentioned above) with tubes through a peristaltic pump to regulate the flow rate. An optional syringe containing sulphide - fixed to a pump regulating the input to the chamber - was connected by tubing to the aquarium. Before the *Z. niveum* colonies or swarmers were placed in the chamber, the system was installed and ran for some time to ensure that the tubes were free of oxygen bubbles and to obtain the correct biotic and abiotic conditions with which to start the treatment. *Z. niveum* colonies were placed in the aquarium by opening the glass plate cover. *Z. niveum* swarmers were injected into the enclosed aquarium with a syringe through an opening in the side of the aquarium, where the in- flow water tubes are normally connected.

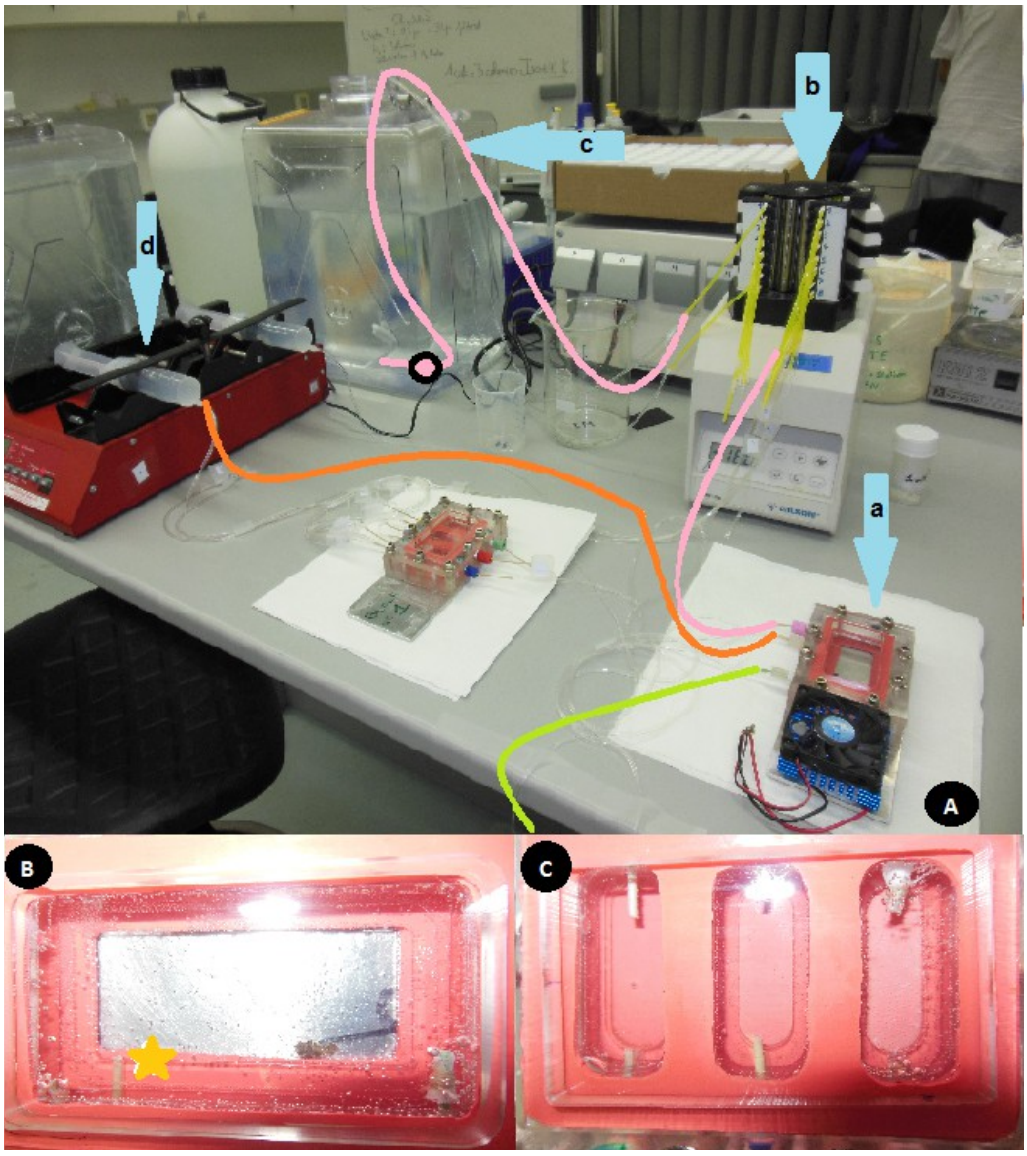


FIG. 3: (A) Composition of the flow-through respirometer system: (a) the acrylic glass aquarium is connected with tubes (follow pink line) to a (c) 20 litre canister containing in-flow water through a (b) peristaltic pump which regulates the flow-rate of the in-flow into the chamber. (d) A syringe containing sulphide solution fixed to a flow-rate regulating pump is connected to the aquarium by tubing (follow orange line). The out-flow water exits through tubing (follow green line) to a bucket at a lower level (not visible in the figure); The aquaria have a volume of either **(B)** 36ml or **(C)** 6 ml, composed of a block of three aquaria beside each other. The sulphide solution enters at ground level (*) in the chamber.

2.3 Monitoring of the chemical parameters

The chemical parameters were measured for all experiments maintained in the flow-through respirometer system at the chamber out-flow (see FIG. 3) every 12 hours. Conductivity, salinity, temperature and pH were measured with a Multi 340i (WTW) sensor. The oxygen saturation rate [%] was estimated with salinity and temperature. Oxygen was measured with a PreSenS Flow-through Cell FTC-PSt3. Sulphide (≅ H_2S: all species of H_2S, HS^-, S^{2-}) was measured using a quantitative colorimetric assay (Cline *et al.* 1969) and a spectrophotometer (Dr.Bruno Lange GmbH, Obergrafendorf, Germany).

2.4 Experimental Setup

Three freshly collected colonies were fixed (*in situ*) immediately after collection from wood sample # 28. Two different approaches were followed to get an insight on changes in the symbionts morphology and fitness resulting from changes in biotic and abiotic environmental parameters under artificial conditions. In one approach adult colonies were treated under sulphide preclusion for 48 hours (Experiment 1 & 2). In the other approach swarmers were used to inoculate a flow-through respirometer acrylic glass aquarium, which ran for 7 days under different biotic and abiotic conditions (Experiment 3,4 & 5).

2.4.1 Experiments obtained with full-grown *Zoothamnium niveum* colonies (FIG. 4)

<u>Experiment 1 under normoxic conditions in the flow-through respirometer system (FLOW)</u>

For the first experiment a total of 70 colonies were used from wood blocks #5 and #6 (Table 1) after they were maintained for 23 days in a 40 litre flow-through container supplied with seawater and the colonies supplied with 300 ml of 2 mM sulphide solution every second day. The colonies were maintained in a 36 ml flow-through respirometer aquarium under normoxic, non-axenic conditions (no sulphide supplied; 32µm filtered surface seawater).

Experiment 2 under stagnant, normoxic conditions (NO FLOW)

Immediately after collecting wood samples #25, #28, #29 in the field (Table 1), colonies were isolated and collected in embryo dishes. Ten embryo dishes with 0.2 µm filtered seawater were prepared with ten colonies per embryo dish and covered with a glass plate to avoid evaporation.

For each experiment 15 colonies were sacrificed every 12 h (t_{12} ,t_{24}, t_{36}, t_{48}, t_{60}), whereby three colonies were fixed for SEM and the remaining 11 colonies were fixed differently. The samples for t_{48} have been used for further investigations, allowing comparison between the two experiments.

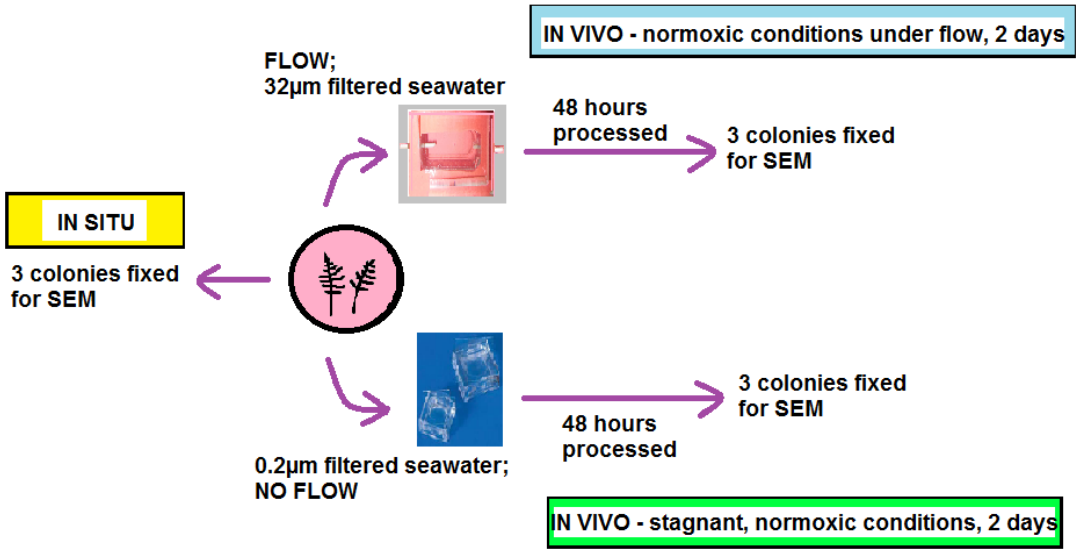


FIG. 4: Composition of experiments obtained with full-grown *Zoothamnium niveum* colonies (Experiments 1 & 2).

2.4.2 Experiments obtained with swarmers of *Zoothamnium niveum* (FIG. 5)

2.4.2.1 Maintenance of swarmers

Swarmers used in these experiments had to be released by adult colonies. Therefore adult colonies with macrozooids attached were pooled after dissection and cleaning, with up to 15 individuals per embryo dish in 0.2 µm filtered, normoxic seawater. Under these conditions the macrozooids were successfully released. Referred to as swarmers, the released macrozooids were pooled in an extra embryo dish. After four to six hours, enough swarmers were released to start a cultivation (pers. data. Volland J.-M., Kolar I., Drexel J. & Espada Hinojosa S., 2012).

2.4.2.2 Inoculation of the aquarium with swarmers

The flow-through respirometer system was ran prior to inoculation to ensure the acrylic glass aquarium did not contain any oxygen bubbles as well as to maintain an accurate concentration of sulphide for settling of the swarmers. During inoculation and settlement, the aquarium was isolated. After settling of the swarmers, the flow-through respirometer system was turned on and the treatment started (pers. data. Volland J.-M., Kolar I., Drexel J. & Espada Hinojosa S., 2012).

2.4.2.3 Conducted experiments

<u>Experiment 3 under optimal, non-axenic conditions in the flow through respirometer system (NONA)</u>

Colonies were used from wood samples #19, #20 and #21 (Table 1) stored for one day in a 40 litre flow-through container supplied with fresh seawater and inoculated with 300 ml of 2 mM sulphide solution. The time to obtain a total of 75 swarmers to inoculate the enclosed aquarium under optimal, non-axenic conditions (10.4 µM sulphide, 32 µm filtered seawater) was not followed exactly. Information on time of settlement is also unavailable. Once the majority of swarmers had settled, the flow-through respirometer system was reinstalled on the

aquarium and ran for seven days under optimal, non-axenic conditions (sulphide supplied; 32 µm filtered surface seawater). During this time, a maximum of 66 colonies were detected. A total of 56 colonies were observed after 7 days, with 25 colonies isolated, whereby three colonies were fixed for analysis with a scanning electron microscope. The remaining colonies were fixed differently for use in other analysis.

Experiment 4 under optimal, axenic conditions in the flow through respirometer system (OPA)

This experiment was conducted in two 6 ml aquaria simultaneously. After settling of the inoculated swarmers, the aquaria were connected to a flow-through respirometer system for seven days under optimal, axenic conditions (sulphide supplied; 0.2 µm filtered surface seawater).

In the first aquarium, colonies were used from wood samples #5 and #6 (Table 1) which were maintained for 18 days in a 40 litre flow-through container supplied with fresh seawater and inoculated with 300 ml of 2 mM sulphide solution every second day. After 10 hours, 61 swarmers were released and the isolated aquarium was inoculated under optimal, axenic conditions (46.9 µM sulphide, 0.2 µm filtered sea water). After 1 h 30 min, 92% of all swarmers were settled and the reinstalled flow-through respirometer system was ran for seven days. During this time a maximum of 18 colonies were detected.

In the second aquarium, colonies were used from wood samples #22, #23, #24, #25, #26, #28, #29 and #30 which were stored for one day in a 40 litre flow-through container supplied with fresh seawater and inoculated with 300 ml of 2 mM sulphide solution. The time to obtain a total of 57 swarmers with which to inoculate the enclosed aquarium under the same conditions as the first aquarium is unknown. After 3 h 15 min, 96% of all swarmers were settled and the reinstalled flow-through respirometer system was ran for seven days. During this time a maximum of 20 colonies were detected. After three days in the flow-through respirometer system all colonies became small and pale, therefore only one colony was used.

A total of 19 colonies were isolated, whereby three colonies were fixed for analysis with a scanning electron microscope. The remaining colonies were fixed differently, to be used for other analysis.

Experiment 5 under normoxic, non-axenic conditions in the flow through respirometer system (NONA)

In this experiment three 6 ml aquaria were ran simultaneously, of which two aquaria were inoculated one day earlier. After settling of the inoculated swarmers, the aquaria were connected to a flow-through respirometer system for seven days under normoxic, non-axenic conditions (no sulphide supplied; 32 µm filtered surface seawater).

The colonies used were taken from wood samples #19 and #20 (Table 1), which were maintained for 2 and 3 days, respectively, in a 40 litre flow-through container supplied with fresh seawater and twice inoculated with 300 ml of 2 mM sulphide solution.

For the first and the second aquarium the time of release of enough swarmers from the dissected colonies for inoculation is unknown. The first enclosed aquarium under optimal, non-axenic conditions (no sulphide supplied; 32 µm filtered surface seawater) was inoculated with 57 swarmers. The second aquarium, under the same conditions, was inoculated with 55 swarmers. In both aquaria 100% of the swarmers were settled after 1 h 45 min.

The third aquarium, under the same conditions as the previous two aquaria, was inoculated with 30 swarmers, which were released from the dissected colonies over 24 hours. The time of settling was not measured. After settling, the flow-through respirometer system was reinstalled and ran for seven days. The maximum number of colonies observed in aquarium one, two and three were 12, 24 and 17 respectively, with a total of 20 colonies isolated after 7 days.

Out of the 20 total colonies, three colonies were fixed for analysis with the scanning electron microscope. The remaining colonies were fixed differently, to be used for other analysis.

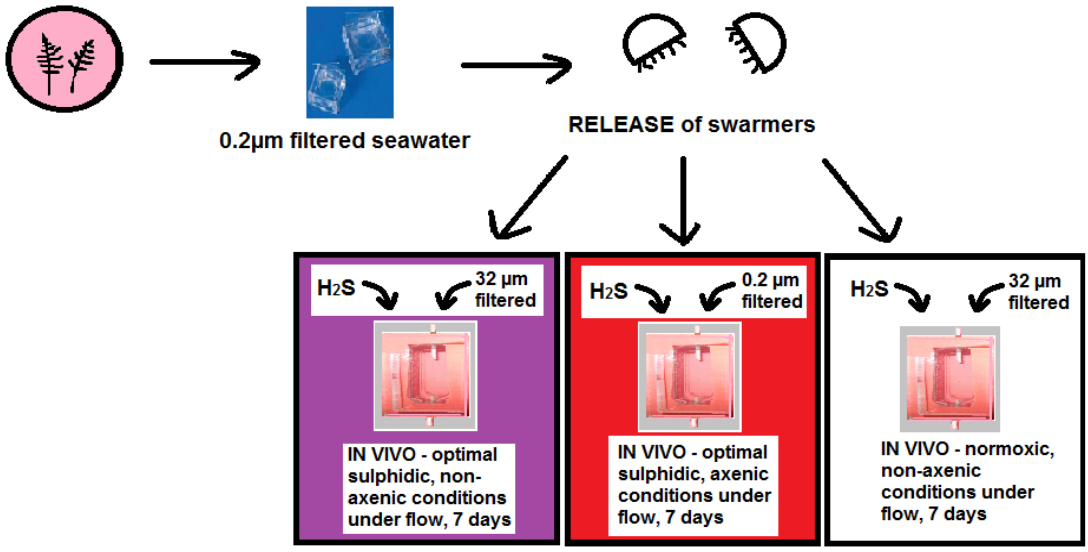


FIG. 5: Composition of experiments obtained with *Zoothamnium niveum* swarmers (Experiments 3,4 & 5).

2.5 Preparation of SEM-samples

To avoid complete contraction of the colonies, which would make the observation of single microzoid difficult, the ciliates were cooled down prior to fixation. Colonies were collected in an embryo dish filled with 2.5 ml of 0.2 µm filtered seawater and put in a freezer at - 20 °C for 9 min 30 sec prior to fixation, according to Rinke *et al.* (2007). Before the freezing point was reached, the embryo dish was taken out and 2.5ml of Trump´s fixative (2.5 % glutaraldehyde, 2 % paraformaldehyde in 0.1 M sodium cacodylate buffer 1100 mOsm, pH 7.2, filtered with a 0.2 µm filter prior to usage) was added (a modification of the method of Trump & Bulger, 1966). The samples were then rinsed with this solution and stored until further treatment. The specimens were further processed upon delivery to the laboratory. Samples were washed in the same buffer three times, for 3 min each time. The samples were then dehydrated through an ascending acetone series (2 times 5 min in 30%, 50%, 70%, 80%, 90% and 3 times 5 min in 100%). The samples were then transferred to a mixture of acetone/hexamethyldisilazane (HDMS) (1:1) for 15 min, followed by two baths of pure HDMS for 15 min each. The samples were subsequently air dried in a clean embryo dish for three hours, placed on a stab and sputter coated with gold-palladium.

2.6 Observation and measured parameters

For each experiment, a triplicate of *Zoothamnium niveum* samples was observed with a Philips XL 20 scanning electron microscope at an acceleration voltage of 20kV. For each sample an image was taken of 15 feeding cells (microzooids) at a magnification of 2000x (see FIG. 6). Each image was analysed, with the microzooids divided in half (perpendicular to the long axis) into an oral and an aboral part and the images used taken from the most oral and most aboral visible areas. For each part several parameters relating to the ectosymbiont covering were evaluated. To analyse the number of cells and the percentage of host surface coverage, a 70µm² rectangular frame was placed at each end of the selected microzooid within the image.

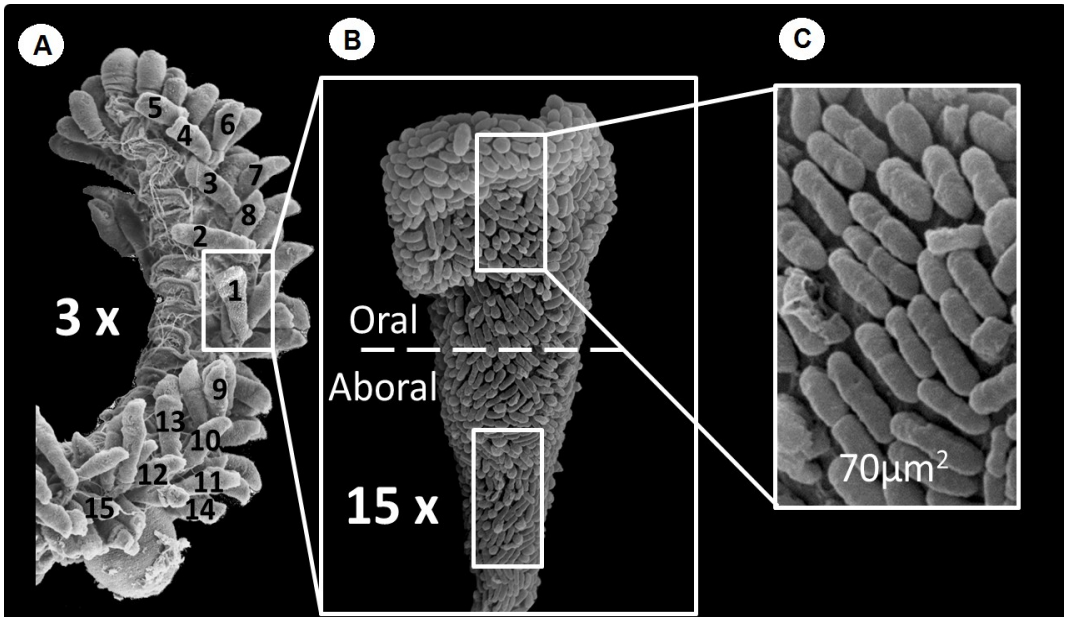


FIG. 6: Analysis of samples: Three samples were analysed per treatment. **(A)** For each sample an image was taken of 15 feeding cells (microzooids) at a magnification of 2000x. **(B)** Each image was analysed, whereby the microzooids were divided in half (perpendicular to the long axis) into an oral and an aboral part. **(C)** A 70µm² rectangular frame was placed at each end of the selected microzooid within the image.

To evaluate the number of cells, all cells extending above the edge of the frame were counted only along one length and width of the frame, while the cells crossing the other two sides were not counted. Cells which crossed the whole frame either longitudinally or horizontally were also counted. The percentage of host surface coverage was measured with Gimp 2.8 software (GNU Image Manipulation Program), whereby all cells were included in the analysis. Thus all cells, even those only found slightly within the frame, were encircled, coloured and the total amount of coloured pixels evaluated and compared with the total amount of pixels within the frame.

The analySIS® program (Soft Imaging System GmbH, Münster, Germany) was used to measure the length and width of up to 70 cells for both parts of the microzooids, with cells selected in a clockwise helical pattern.

The length and width data was used to calculate the volume of the symbionts. Each symbiont was considered as a cylinder with two hemispheres and the volume was measured by ´adding the volumes of the two half-spheres with radium equal to 0.5x the width of the rod, plus length equal to the length of the rod minus 2x the radium of the half-spheres´ (van Veen & Paul, 1979).

The cell elongation index (CI), the ratio of length to width, was calculated for each cell. Cells with a CI of approximately 1 were considered coccoid-shaped, while cells with a CI greater than 1.5 were considered rod-shaped (Sunamara *et al.* 2004).

The frequency of dividing cells (FDC) – calculated as ((mean number of dividing cells/mean number of total cells) x 100) - was measured in the same manner by counting clockwise helical up to 70 cells. Dividing cells are defined as bacteria showing an invagination but not a clear intervening zone between cells (Hagström *et al.* 1979). The absolute number of dividing cells was evaluated per 70 µm² (= (FDC x N° of cells per 70µm²)).

2.7 Statistics

All parameters were tested with IBM® SPSS® Statistics 20. Within all treatments, the oral parts of the microzooids were tested against the aboral parts. Between all treatments, the oral and the aboral parts were tested separately, with the total microzooids also tested. As the Kolmogorov-Smirnov test with Lilliefors correction of significance did not indicate a normal distribution for the parameters tested within each group, non-parametric statistics were used. When two parameters were tested against each other the Mann-Whitney U-test with a 99% CI was applied. When more than two parameters were tested against each other the Kruskal-Wallis H-test with a 99% CI and the multiple comparison Tamhane *post hoc* test with a 99% CI for unequal variances were used.

3 RESULTS

Detailed values for chemical conditions and measured parameters are given in Appendix (7.1 & 7.2)

3.1 Morphology and fitness of *Cand.* Thiobios zoothamnicoli *in situ* (see FIG. 9 (A))

3.1.1 Measured parameters

3.1.1.1 Morphology (see FIG. 13 & 14)

The CI indicated that the ectosymbionts are significantly more coccoid-shaped on the oral parts (2.16 $\pm$ 0.68; N = 3150) and more rod-shaped on the aboral parts (2.54 $\pm$ 0.9 ; N = 3088). The values for length and width were also significantly higher on the oral part, resulting in significantly more voluminous ectosymbionts on the oral part (26.04 $\pm$ 12.66 µm³; N = 3150) than on the aboral part (10.9 $\pm$ 7.54 µm³; N = 3088).

3.1.1.2 Fitness (see FIG. 15 & 16)

The number of cells was significantly lower on the oral parts compared to the aboral parts. However, the percentage of host surface coverage was equally high on both parts (88.98 $\pm$ 3.09 %; N = 90).

The FDC of ectosymbionts covering the aboral part of the microzooids was significantly lower than for those covering the oral part. However, the absolute number of dividing cells / 70 µm² was equal for both parts (10.22 $\pm$ 2.74; N = 90).

3.2 Changes in morphology and fitness of *Cand.* Thiobios zoothamnicoli due to changes of biotic or abiotic conditions *in vivo*

3.2.1 Presence/absence of free-living microbes

3.2.1.1 Chemical conditions

Swarmers used for the OPNA experiment were treated for 7 days under optimal, non-axenic conditions (sulphide supplied; 32 µm filtered surface seawater). The pH and salinity remained stable, while the temperature and flow-rate showed variations. The oxygen concentration (254.03 ± 16.77 µmol/L; *N* = 14) decreased over the time of measurement from 268.3 µmol/L to 225 µmol/L - giving an oxygen saturation of 119.4 % to 102 % respectively - with fluctuations over this period. The sulphide concentration data (16.33 ± 21.94 µM; *N* = 14) was highly variable, with measured values varying between undetectable and a maximum of 63.96 µM (see FIG. 7 (a)).

Swarmers used for the OPA experiment were treated for 7 days under optimal, axenic conditions (sulphide supplied; 0.2 µm filtered surface seawater). For this experiment two aquaria were used simultaneously. For both aquaria, the pH and salinity remained stable over the duration of the experiment, while the temperature and flow-rate showed variations. Both aquaria had fluctuating oxygen concentrations over 110% of the oxygen saturation rate – for which the data logging was incomplete - with greater fluctuations in one aquarium (264.19 + 12.49 µmol/L; *N* = 7). The same aquarium had greater fluctuations in sulphide concentration (18.98 ± 9.48 µM; *N* = 11), with a minimum of 2.25 µM and a maximum of 37.29 µM (see FIG. 7 (b)), however, these measurements still reside in the estimated optimal range for sulphide concentration (Rinke *et al.* 2007).

3.2.1.2 Measured parameters

3.2.1.2.1 Morphology (see FIG. 13 & 14)

Ectosymbionts covering colonies from the OPNA (see FIG. 9 (B)) and OPA (see FIG. 9 (C)) treatments were seen to have the same trend in CI and volume as the ectosymbionts covering the *in situ* microzooids: more coccoid-shaped and more voluminous on the oral parts than

on the aboral parts of the microzooids. However, the ectosymbionts covering the oral parts of the microzooids of both experimental treatments were significantly more rod-shaped compared to the *in situ* situation, with those under OPA conditions having the highest CI value. Ectosymbionts treated under OPNA conditions were seen to be significantly longer and therefore significantly more voluminous (2.38 ± 1.31 µm²; *N* = 5589) compared to ectosymbionts treated under OPA conditions (1.53 ± 1.43 µm²; *N* = 4932) and the *in situ* colonies. Ectosymbionts treated under OPA conditions had a significantly lower volume on the oral part compared to the *in situ* situation, as they were narrower, while those on the aboral part had a significantly higher volume as they were broader. When the total microzooids were compared, however, the OPA ectosymbionts had a significantly lower volume than the *in situ* situation.

3.2.1.2.2 Fitness (see FIG. 15, 16 & 17)

A comparison of both treatments showed that the OPA colonies had a significantly higher number of ectosymbionts on the oral part than those treated under OPNA conditions, with OPA values equal to the *in situ* values. The number of cells on the aboral parts were similar for both treatments and significantly lower than for the *in situ* situations. However, the percentage of host surface coverage was equally high for both parts within both experimental treatments, with these values identical to the *in situ* situation.

Within the treatments, the FDC of the OPNA ectosymbionts was significantly higher on the oral parts compared to the aboral parts, while no significant difference was seen for the OPA treatment. No significant differences were found when the treatments were compared to each other and the *in situ* situation. In contrast, no significant difference was seen in the absolute number of dividing cells / 70 µm² when the oral and the aboral parts are compared with each other within each treatment. When both treatments were compared, OPNA (7.79 ± 2.12; *N* = 90) and OPA (8.68 ± 3.82; *N* = 90) were found to be significantly lower than the *in situ* situation. When the two artificial treatments were compared to each other, again, no significant difference in the absolute number of dividing cells / 70 µm² was seen.

3.2.2 Sulphide preclusion

3.2.2.1 Experiments undertaken with colonies

3.2.2.1.1 Chemical conditions

For the FLOW experiment, *Zoothamnium niveum* colonies with *Cand.* Thiobios zoothamnicoli attached were maintained in a flow-through respirometer aquarium (Rinke *et al.* 2007) under normoxic, non-axenic conditions (no sulphide supplied; 32 µm filtered surface seawater). Over the 48 hour duration of the experiment, the pH, temperature, salinity and flow-rate remained stable. The oxygen concentration ranged from 252.8 to 267.9 µmol/L (N = 4), giving an oxygen saturation rate of 117.57 $\pm$ 3.64 %, with sulphide not detected (see FIG. 8 (a)). For the NO FLOW treatment, the chemical conditions were not monitored over the duration of experiment as the colonies were maintained in embryo dishes.

3.2.2.1.2 Measured parameters

3.2.2.1.2.1 Morphology (see FIG. 13 & 14)

The ectosymbiont cells covering colonies under FLOW (see FIG. 10 (A)) and NO FLOW conditions (see FIG. 10 (B)) both showed a tremendous increase in length as compared to the *in situ* scenario, with the largest increase for those covering the FLOW colonies. On colonies treated under FLOW conditions, filamentous bacteria exhibiting small bulges were observed mainly on the aboral part of the microzooids (see FIG. 11). Ectosymbionts were seen to be broadest on the NO FLOW colonies. These findings resulted in a significant increase in volume and CI for both artificial treatments, with most voluminous ectosymbionts seen on the NO FLOW colonies and the most rod-shaped ectosymbionts seen covering the FLOW colonies. The trend in CI and volume for both artificial treatments was seen to be the same as for the ectosymbionts covering the in *situ* microzooids: more coccoid-shaped and voluminous on the oral parts than on the aboral parts of the microzooids.

3.2.2.1.2.2 Fitness (see FIG. 15, 16 & 17)

A significant decrease in number of cells and percentage of host surface coverage was seen for both artificial treatments compared to the *in situ* situation, with a larger decrease for the NO FLOW treatment overall. Comparisons of the two artificial treatments showed a significantly lower amount of cells and percentage of host surface coverage for the NO FLOW treatment on the oral parts of the microzooids, but both parameters were not significantly different on the aboral parts.

In contrast to the *in situ* situation, both artificial treatments did not show a difference in FDC when their oral parts were compared to their aboral parts. However, the absolute number of dividing cells / 70 µm² was significantly lower on the oral parts as compared to the aboral parts. While the FDC was seen to be significantly lower only on the oral parts when compared to the *in situ* situation, the absolute number of dividing cells / 70µm² was seen to be significantly lower for both parts of the microzooids, with the significantly lowest values for the NO FLOW treatment.

3.2.2.2 Experiment undertaken with swarmers

3.2.2.2.1 Chemical conditions

Swarmers used for the NONA (see FIG. 12) experiment where treated for 7 days under normoxic, non-axenic conditions (no sulphide supplied; 32 µm filtered surface seawater). For this experiment three aquaria were used simultaneously. For all three aquaria the pH and salinity remained stable, while the temperature, flow-rate and oxygen concentration varied. The measured mean values for oxygen concentration and saturation rate were equal in all three aquaria, with highest variations in aquarium N°3 (276.29 $\pm$ 9.3 µmol/l; 123.28 $\pm$ 2.3 %) (see FIG. 8 (b)). Sulphide was not detected in all three aquaria.

3.2.2.2.2 Measured parameters

The colonies obtained from swarmers treated under normoxic, non-axenic conditions for 7 days (NONA) were aposymbiotic. Therefore no parameters could be measured: the fitness of

the ectosymbionts was zero. When the colonies were isolated from the chambers they already appeared pale in contrast to the other colonies treated under OPNA and OPA conditions, which appeared bright white.

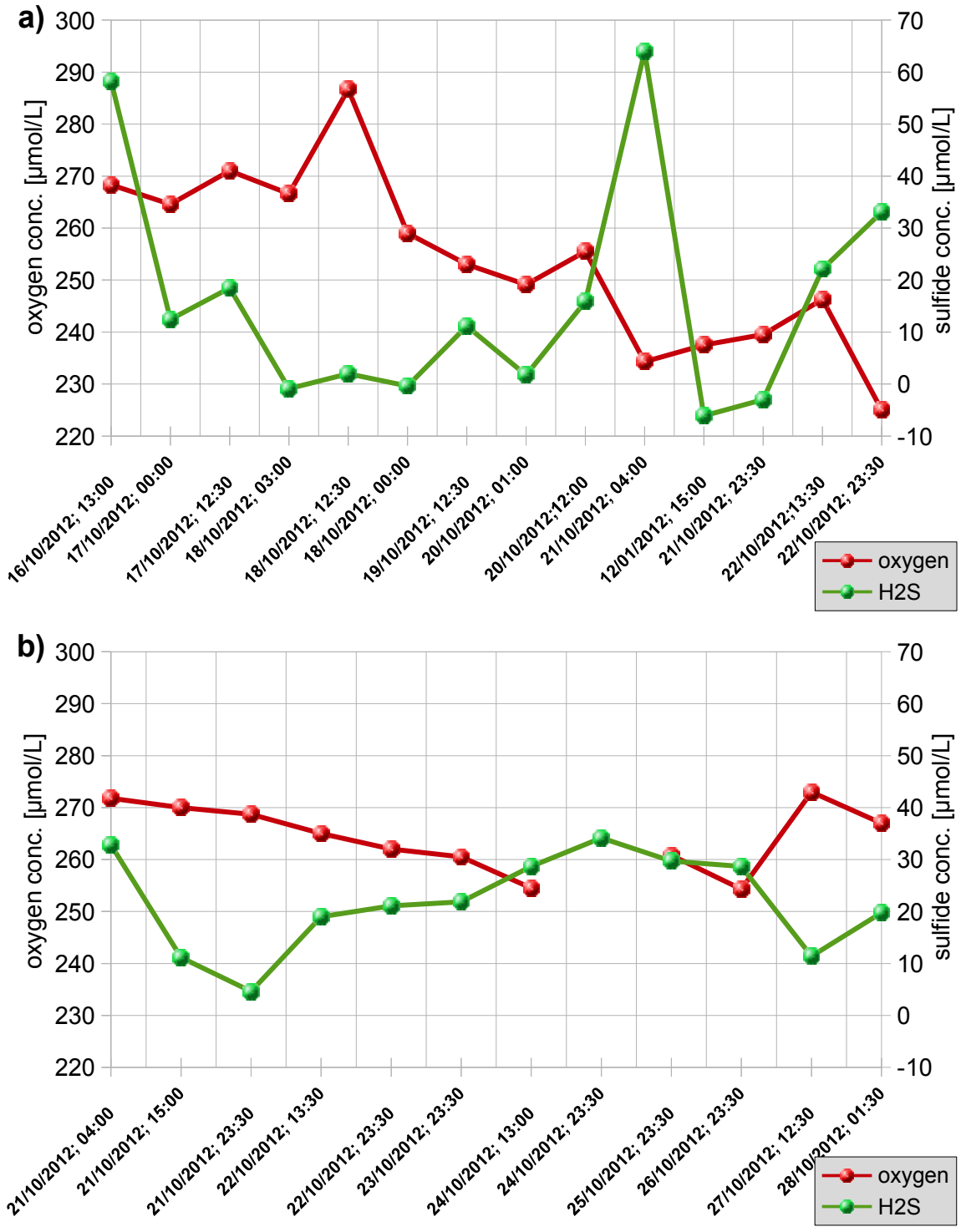


FIG 7. Oxygen concentration [µmol / L] of **a)** the treatment: *in vivo* - under optimal, non-axenic conditions in the flow through respirometer system, 7 days (OPNA). **b) aquaria N° 1** of the treatment: *in vivo* - under optimal, axenic conditions in the flow through respirometer system,7 days (OPA).

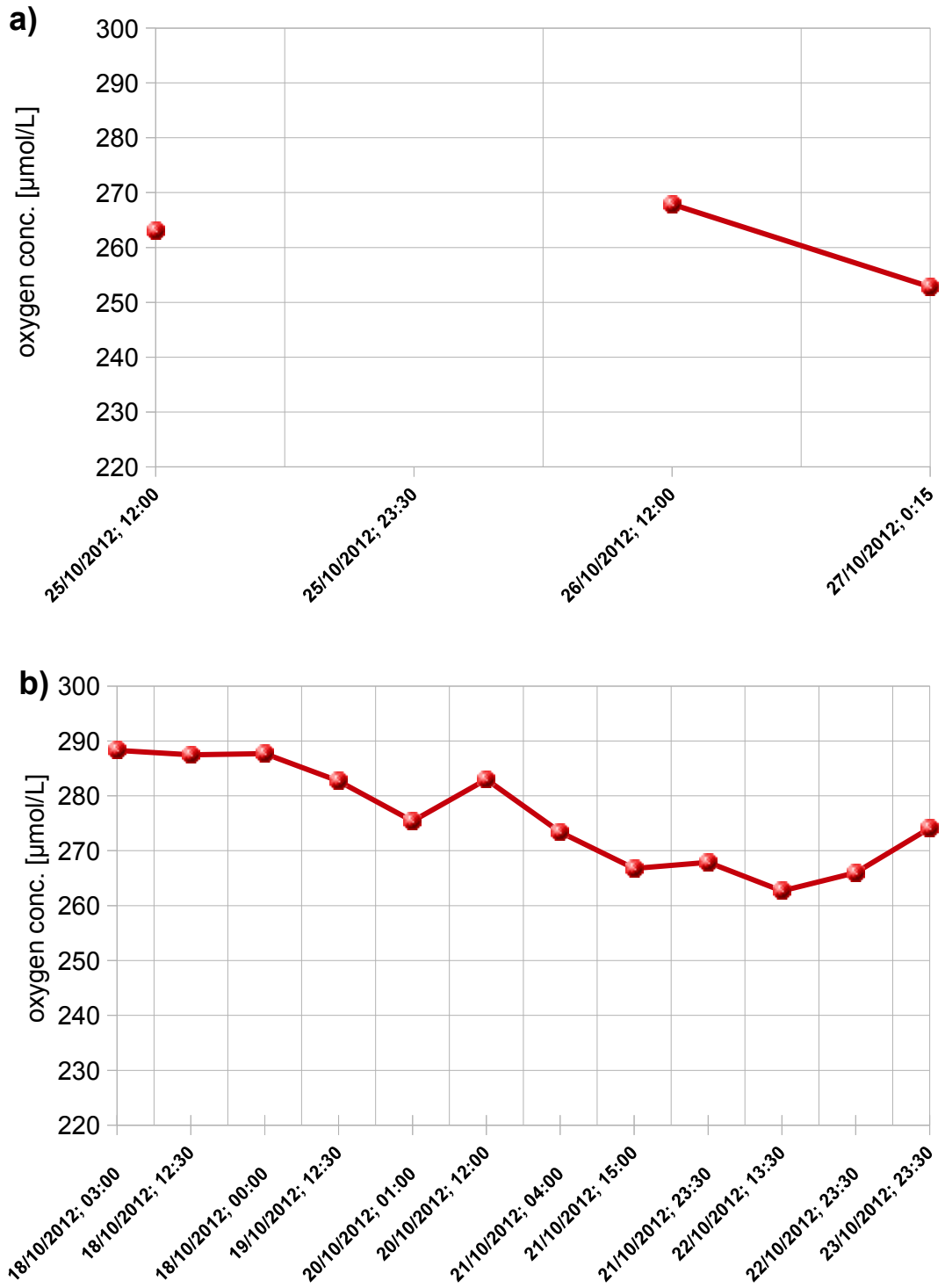


FIG 8. Oxygen concentration [µmol / L] and sulfide concentration [µmol / L] of **a)** the treatment: under normoxic conditions in the flow through respirometer system for 2 days (FLOW). **b) aquaria N° 2** of the treatment: *in vivo* - under normoxic, non-axenic conditions in the flow through respirometer system, 7 days (NONA).

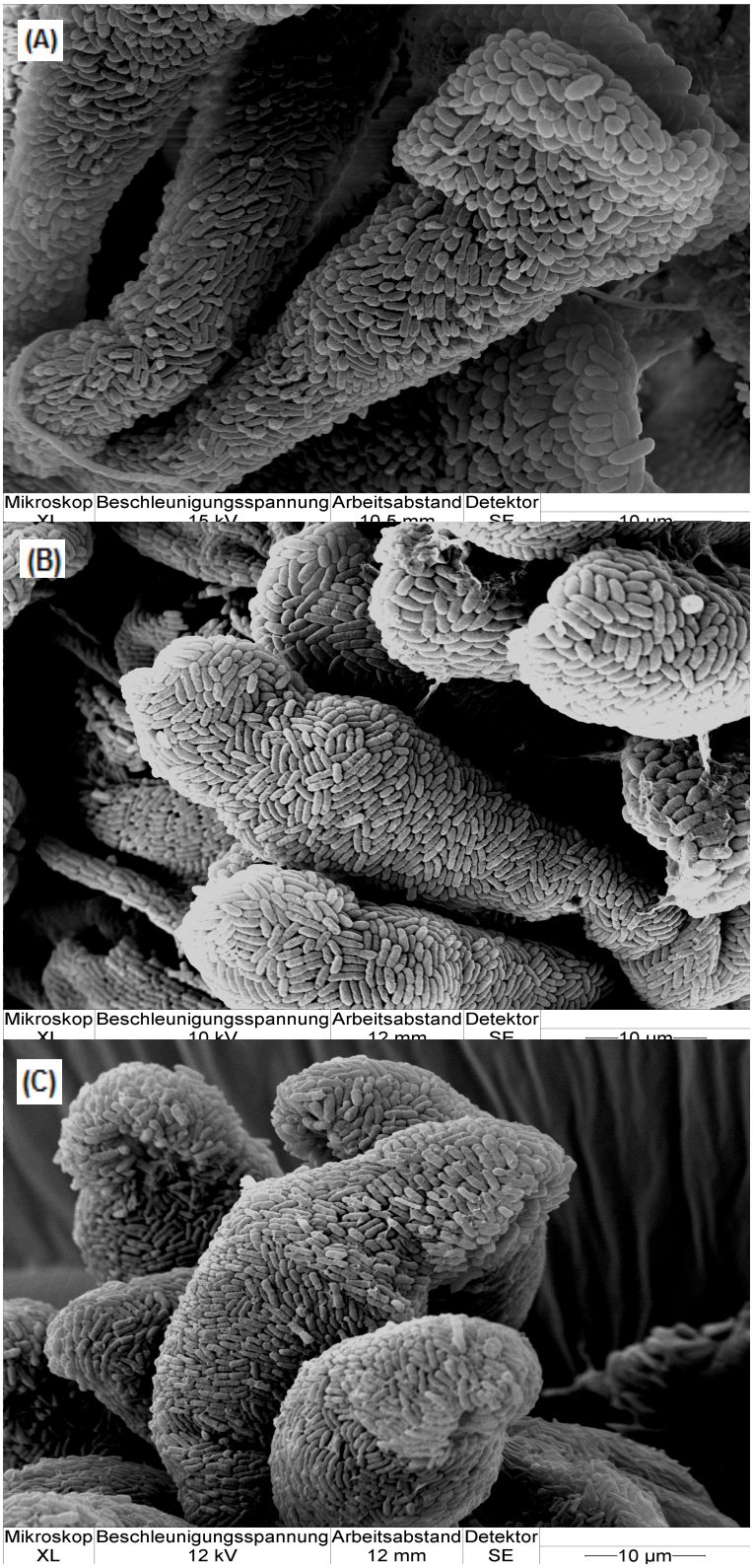


FIG. 9. SEM of *Zoothamnium niveum* microzooids with *Cand.* Thiobios zoothamnicoli attached. **(A)** *in situ*. **(B)** *in vivo* – optimal sulfidic, non-axenic conditions under flow, 7 days. **(C)** *in vivo* – optimal sulfidic, axenic conditions under flow, 7 days.

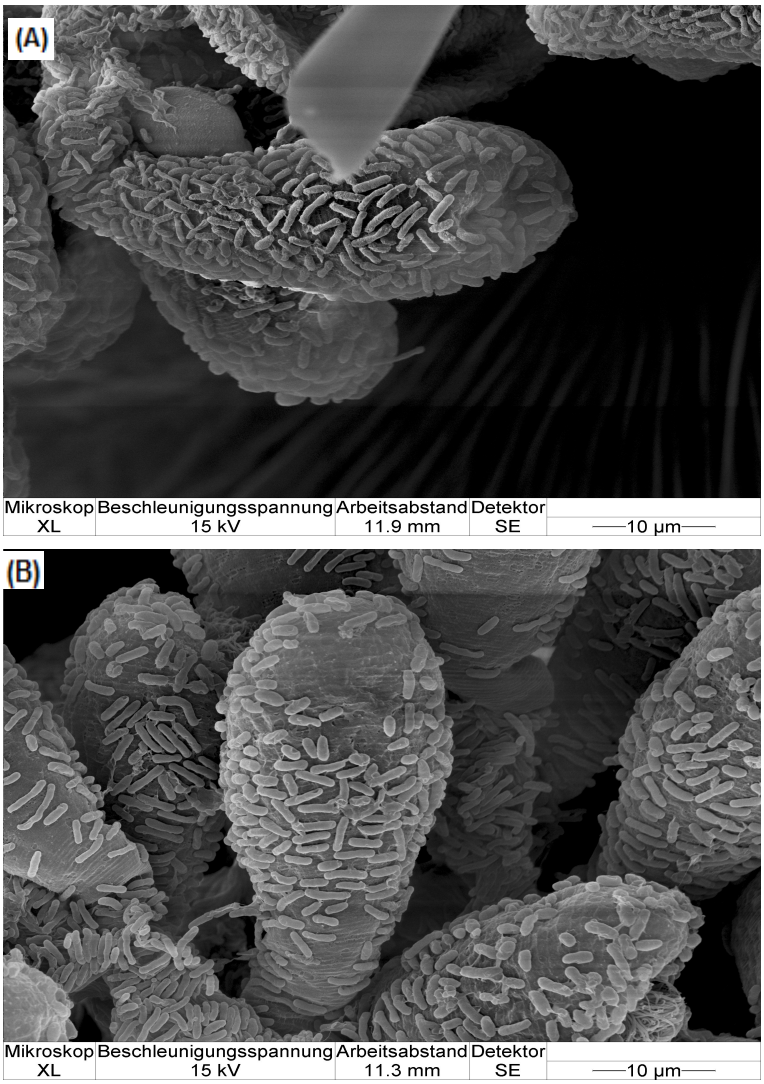


FIG 10. SEM of *Zoothamnium niveum* microzooids with *Cand.* Thiobios zoothamnicoli attached. **(A)** *in vivo* – normoxic conditions under flow, 2 days. **(B)** *in vivo* – stagnant, normoxic conditions, 2 days

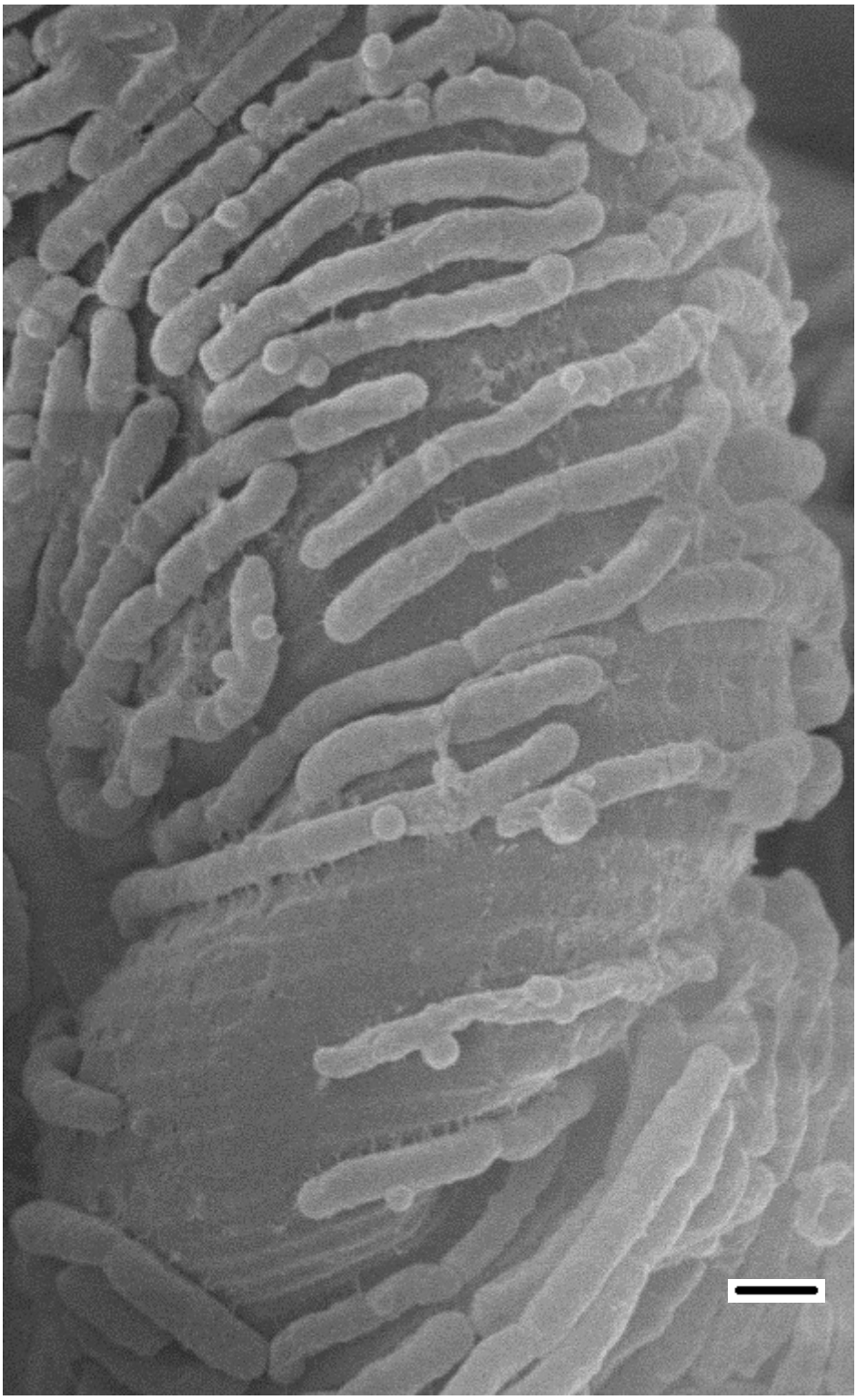
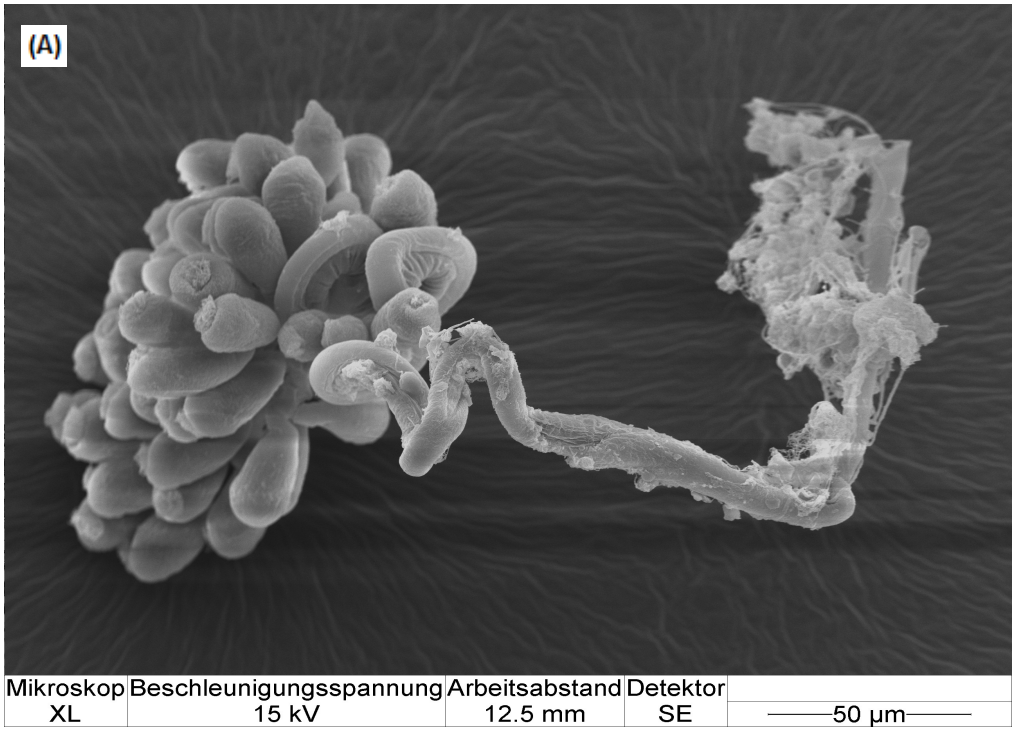


FIG 11. SEM of *Zoothamnium niveum* microzooid attached with filamentous bacteria containing bulges of the *in vivo* – normoxic conditions under flow, 2 days treatment. Bar lengths, 1 μm.



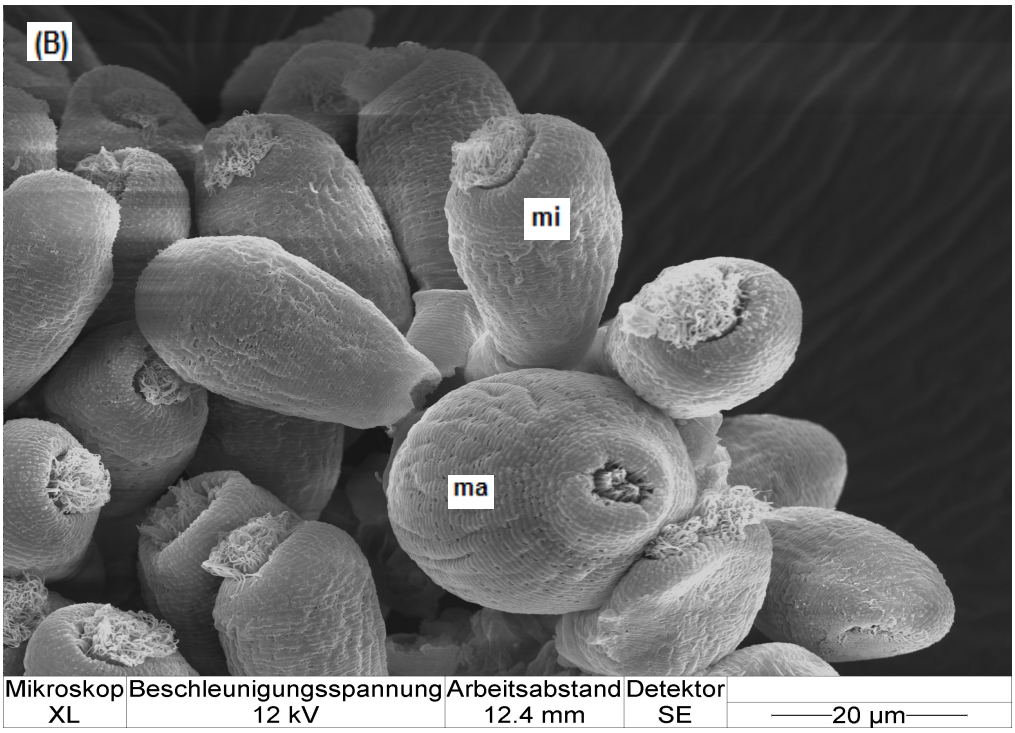


FIG 12. SEM of *Zoothamnium niveum* of the treatment: *in vivo* – normoxic, non-axenic conditions under flow, 7 days. (A) total *Zoothamnium niveum* colony. (B) Detail of the colony, demonstrating two different cell types: macrozooids (ma), and microzooids (mi).

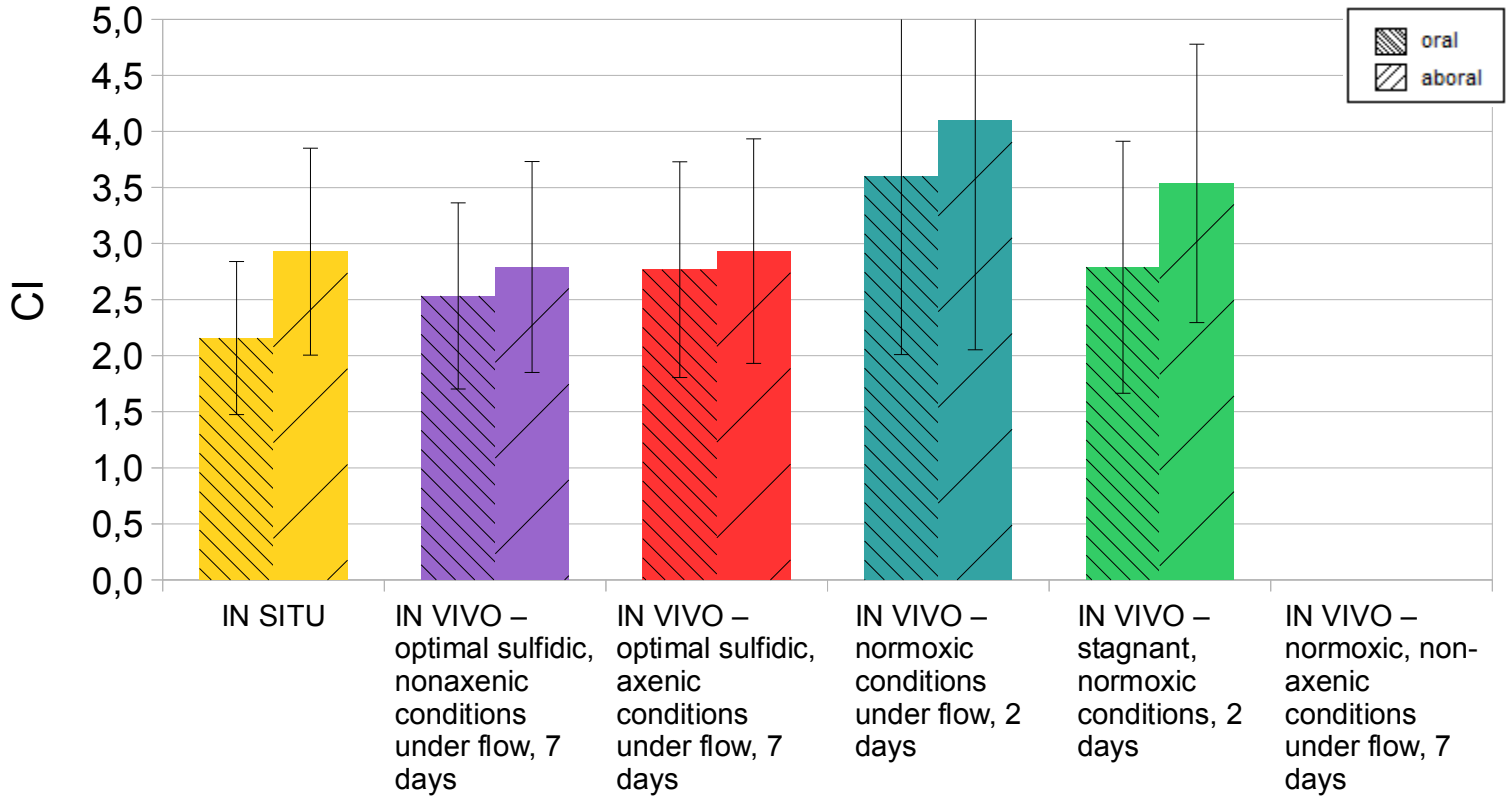


FIG 13. Cell elongation index (CI) of *in situ* and all *in vivo* treatments – Comparison oral vs. aboral part of the microzooids

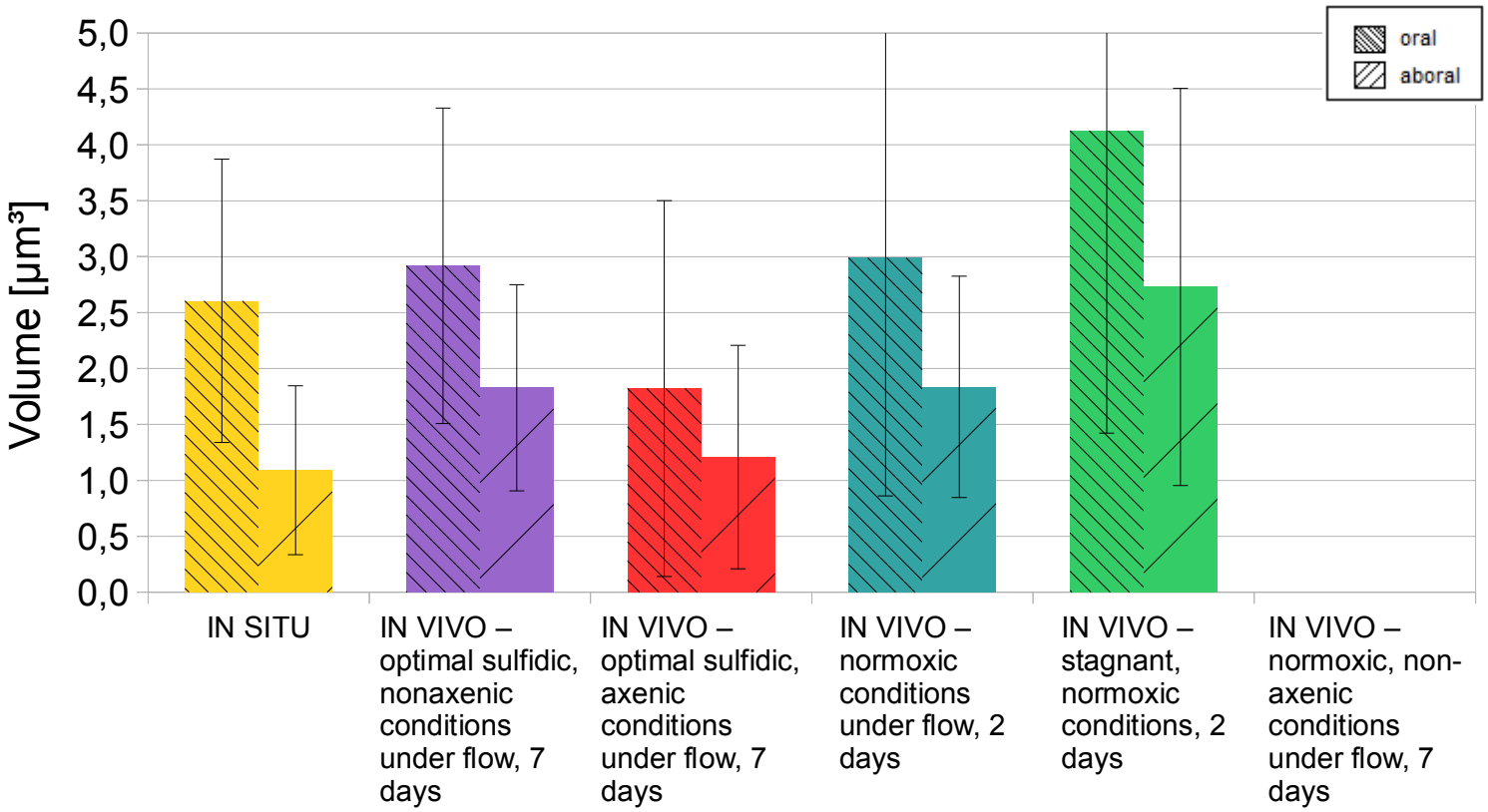


FIG 14. Volume [µm³] of *in situ* and all *in vivo* treatments – Comparison oral vs. aboral part of the microzooids

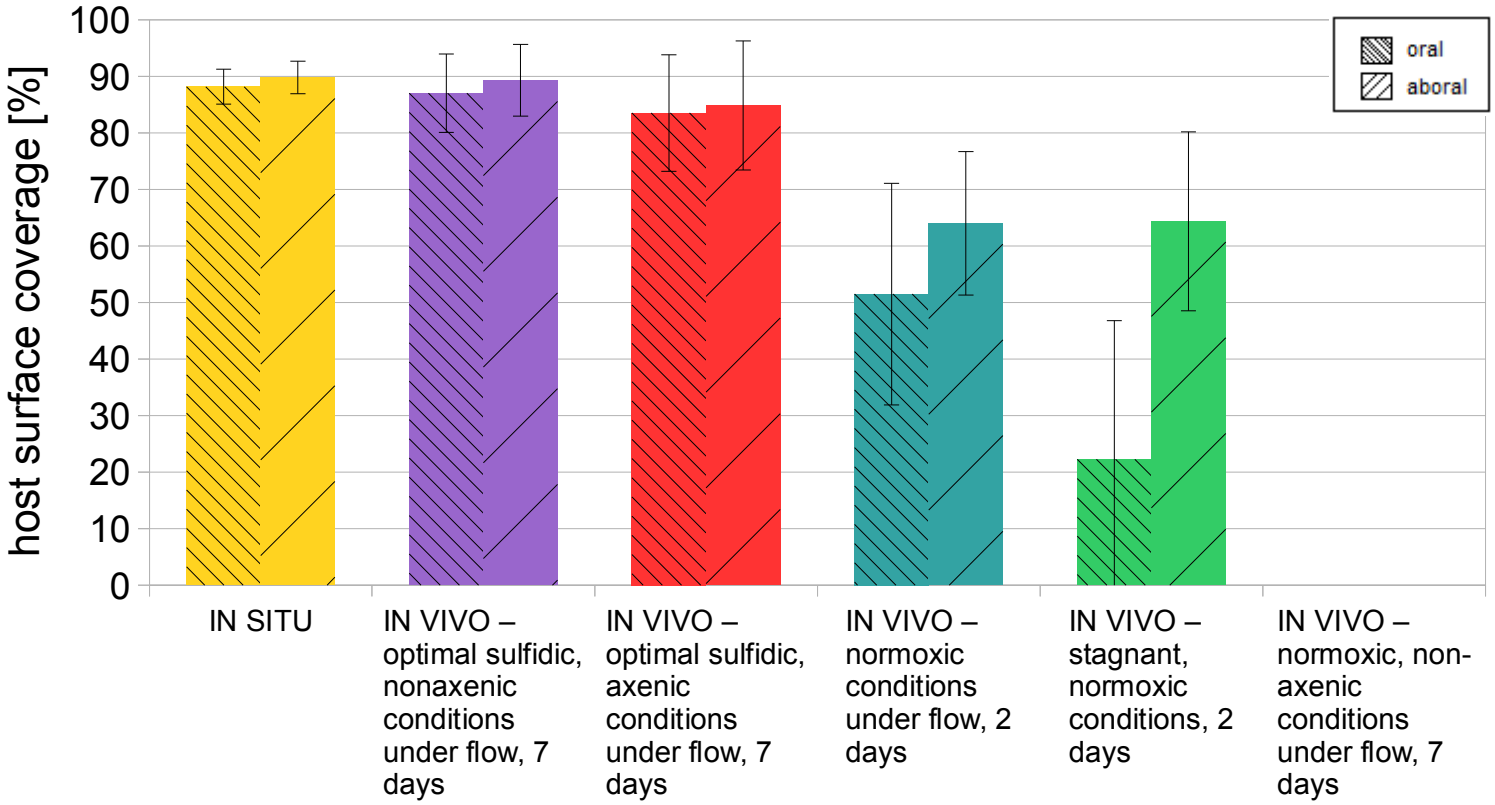


FIG 15. Percentage of host surface coverage [%] of *in situ* and all *in vivo* treatments – Comparison oral vs. aboral part of the microzooids

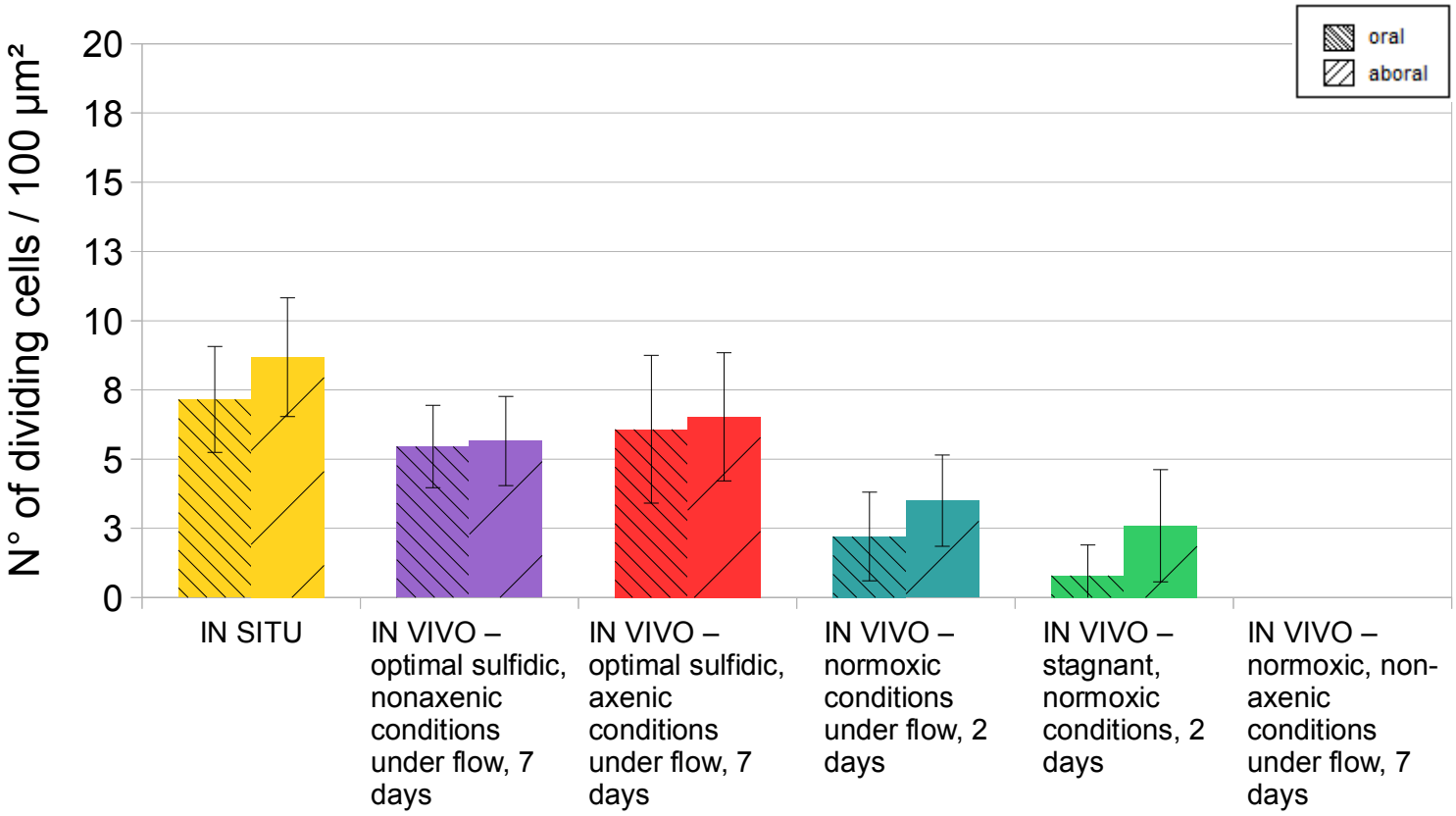


FIG 16. Absolute N° of dividing cells / 100 µm² of *in situ* and all *in vivo* treatments – Comparison oral vs. aboral part of the microzooids

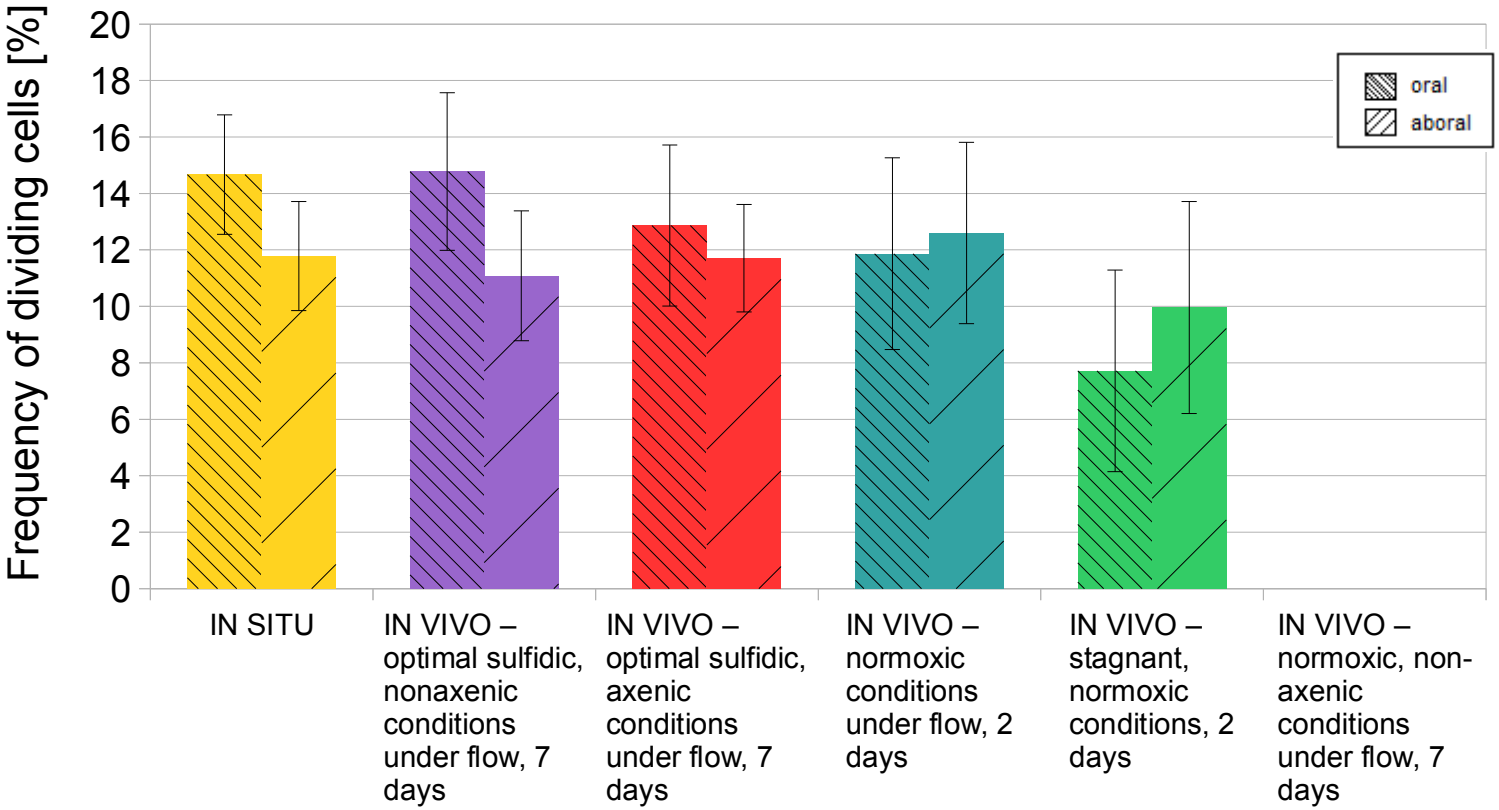


FIG 17. Frequency of dividing cells [%] of *in situ* and all *in vivo* treatments – Comparison oral vs. aboral part of the microzooids

4 DISCUSSION

The *in situ* and the OPNA treatment showed equal trends in the distribution of morphology and fitness parameters from the oral to the aboral part of the microzooid. However, differences appeared when the actual mean values were compared. When the OPNA samples got compared with those treated under OPA conditions, *Z. niveum* showed an approx. 50% lower proliferation rate and therefore size under OPA conditions (pers. data. Volland J.-M., Kolar I., Drexel J. & Espada Hinojosa S., 2012). However, *Cand.* Thiobios zoothamnicoli showed a similar composition on the host surface, although the division rate under both conditions was similar. Under FLOW and NO FLOW conditions the *Cand.* Thiobios zoothamnicoli population size significantly decreased in number. However, the symbiosis was still maintained after 48 hours. The remaining ectosymbionts covering *Z. niveum* samples of both treatments were larger in volume and still demonstrated division. In addition, bulging filaments have been observed on samples treated under FLOW conditions. The established colonies treated under NONA conditions were aposymbiotic. Therefore no parameters could be measured: the fitness of the ectosymbionts was zero.

The results we got from the *in situ* and OPNA treatment are interpreted as a response to the actual chemical conditions to which the samples were exposed and further support the classification of *Cand.* Thiobios zoothamnicoli as a pleomorphic cell-type (Bauer-Nebelsick *et al.* 1996a,b; Rinke *et al.* 2006). In addition, the observed bulging filaments of the FLOW treatment are thought to represent an extreme morphotype of *Cand.* Thiobios zoothamnicoli. The comparison of the OPNA and OPA treatments led to the assumption, that the *Z. niveum* is able to control the population size of its ectosymbiont, to prevent itself from getting overgrown. Due to the results from the FLOW and NO FLOW treatment we suppose that *Cand.* Thiobios zoothamnicoli is capable to utilize nutritional sources other than sulphide. The colonies we obtained from the NONA treatment constitute the first time aposymbiotic colonies could be established under artificial conditions.

4.1 Response to chemical conditions – Pleomorphism

Differences in morphology and fitness of *Cand.* Thiobios zoothamnicoli existed *in situ* as well as under optimal *in vivo* conditions, which was interpreted as a response to the actual chemical conditions to which it was exposed. This finding further supports the classification of *Cand.* Thiobios zoothamnicoli as a pleomorphic cell-type (Bauer-Nebelsick *et al.* 1996a,b; Rinke *et al.* 2006). In addition, *Cand.* Thiobios zoothamnicoli showed extreme morphological changes under sulphide starvation and it was supposed that this cell type is capable of forming bulging filaments.

The ectosymbionts from our *in situ* samples collected from wood pieces in Piran, Slovenia and the ectosymbionts covering the microzooids from our OPNA samples were compared to each other as well as to the *in situ* and optimal *in vivo* samples from Calvi, Corsica, France (Rinke *et al.* 2007). Similar trends in the measured characteristics were seen for all sample sets. The length, width and volume significantly decreased from the oral to the aboral part of the microzooids, while the CI and the FDC significantly increased.

However, differences could be seen between the mean values calculated for the sample sets. The ectosymbionts from our *in situ* samples had a much higher volume and CI – and therefore were more rod-shaped – than those from the Calvi samples. The largest ectosymbionts from the Calvi *in situ* samples were those of the most apical part of the microzooid with a volume of 1.295 + 0.674 µm³ (N = 1800), which was less than half the volume of the ectosymbionts from our *in situ* colonies. The FDC indicated a higher fitness of the ectosymbionts covering the *in situ* samples from Piran than those from Calvi. Differences were also seen when the optimal *in vivo* samples were compared to the corresponding *in situ* situation. Ectosymbionts covering the microzooids from our OPNA samples were significantly more voluminous and more rod-shaped on the oral parts, but less rod-shaped on the aboral parts of the microzooids. In contrast, the *in vivo* ectosymbionts from Calvi were seen to have smaller and more rod-shaped ectosymbionts on all parts of the microzooids. While the Calvi *in vivo* ectosymbionts had a significantly higher FDC on the oral part compared to the corresponding *in situ* situation, the FDC of OPNA ectosymbionts was equal to its corresponding *in situ* situation for the entire microzooid.

A distinction on strain level has already been proven between *Cand.* Thiobios zoothamnicoli populations from seagrass debris at Calvi, Corsica, mangrove peats at Twin Keys, Belize and a deployed whale bone at a depth of 5m in Tokyo Bay (Rinke *et al.* 2009, Kawato *et al.* 2010). It is still to be determined if the *Z. niveum* ectosymbionts from Piran, Slovenia are also distinct to those from Calvi. However, a genetic difference may not be the reason for the differences in morphology and fitness evaluated between the samples from this two different locations as differences have also been observed between our *in situ* and OPNA samples as well as when the *in situ* and optimal *in vivo* samples from Calvi, Corsica were compared (Rinke *et al.* 2007).

The differences observed most likely derive from the different chemical properties to which the samples were exposed. The chemical and physical conditions of our *in situ* samples were not fully characterized i.e. values for sulphide concentrations on the wood surface were unavailable. However, submerged pieces of wood have been seen to be a stable, long lasting habitat for *Z. niveum* symbiosis for more than 80 days, exposed at times to sulphide concentrations of more than 100 µmol L^{-1} (Laurent *et al.* 2013). The *in situ* samples from Calvi derived from patches on *Posidonia oceanica* leaves, where *Z. niveum* patches existed for approximately one month, exposed to sulphide concentrations of between 12 and 1194 µmol L^{-1} over three days of observation (Rinke *et al.* 2007). While the sulphide supply for the Calvi *in vivo* colonies remained between 3 and 33 µmol L^{-1} over the 14 days (Rinke *et. al.* 2007), colonies treated under OPNA conditions were exposed to sulphide fluctuations, with values ranging from undetectable to a maximum of 63.96 µM. Furthermore, our OPNA aquarium was supplied with 32 µm filtered seawater, while 100 µm filtered seawater was used in the Calvi *in vivo* treatment.

The equal trends in the measured morphology and fitness parameters from the oral to the aboral part of the microzooids on our samples from Piran compose the second sample set this was evaluated after the equal trends observed on the samples from Calvi, Corsica (Rinke *et al.* 2007). This supports the claim made after the analysis of the Calvi samples, that *Cand.* Thiobios zoothamicoli responds to chemical variations on micro-scale level, enhanced by the beating of the host´s cilia, which produce a different chemical composition for the ectosymbionts covering the oral part of the microzooids to those covering the rest of the host´s surface.

It is known that the polymorphism of certain free-living bacteria is related to nutrition availability (see White, 2000). As recognized by Rinke *et al.* (2007), the morphology and fitness of *Cand.* Thiobios zoothamnicoli is influenced by the chemical conditions to which it is exposed under optimal *in vivo* and *in situ* conditions. This supports the classification of *Cand.* Thiobios zoothamnicoli as a pleomorphic cell-type (Bauer-Nebelsick *et al.* 1996a,b; Rinke *et al.* 2006).

In addition to the variations in morphology and fitness of the ectosymbionts covering the *in situ* and optimal *in vivo* samples mentioned above, certain samples treated under FLOW conditions – and therefore under sulphide starvation - were found to be covered in bulging, multiple dividing filaments. These cells are thought to be *Cand.* Thiobios zoothamnicoli as a slight transition from long single cells to these bulging filaments is visible through comparison of different cells from the same sample. However, this has still to be proven on genome level by 16S rRNA analysis.

A comparable cell morphology has been observed for *E. coli* cells, where filaments with bulging tips were established after 5 days of starvation through cultivation on silicon wafers, titanium, glass and plastic discs. This filamentation apparently derived from rods which were unable to divide and after two days of observation the cells had built cell-pairs, within which cross-walls were present. The cell filamentation derived from starvation and was independent of the material on which they were cultured, but the nature of and possible reason for bulge formation was unknown (Wainwright *et al.* 1999). In addition to this heterotrophic organism, another microorganism able to change the morphology as a response to chemical conditions is seen with the marine chemolithotroph bacterium *Thiomicrospira thyasirae*, which is capable of growing autotrophically on inorganic sulphur compounds. This gram-negative bacterium is mainly straight- and bent- rod-shaped when cultured on tetrathionate and $NaHCO_3$. However, when cultured on thiosulphate-agar slants, the amount of short spiral forms increases, and when cultured in a tetrathionate-limited chemostat culture, this bacterium appears mainly bent-rod shaped and in spirals of various lengths (Wood & Kelly, 1993).

Thiomicrospira thyasirae is therefore a pleomorphic cell-type, showing extreme differences in morphology when exposed to different chemical conditions (Wood & Kelly, 1993). Another pleomorphic cell-type is the thiotrophic symbiont – an ε-Proteobacterium - of the shrimp *Rimicaris exoculata*, which appears as rods and as filaments of varying width (see Bright, 2002).

If these bulging filaments represent a further type of morphology *Cand.* Thiobios zoothamnicoli can take on due to the chemical exposure conditions, this would widen the range of known cell-shapes for this organism.

4.2 Host control of symbiont population size

Further studies investigating optimal and starvation conditions for the host *in vivo* indicated that the symbiont population size is limited by the proliferation rate and therefore by the size of the host. Although sulphide was available in both conditions, and both symbiont populations showed an equal fitness, the smaller hosts receaved from the OPA treatment were not overgrown by the symbionts. However, upon the death of the host, the symbiont was observed to overgrow the host, as seen in other experiments (pers. observ.). Therefore the factor controlling the homeostasis between the two partners of this symbiosis is suspected to stem from the host, as supported by other examples of symbiosis in literature (see Douglas, 2010; Brooks & Richards, 1955; Ruby and Asato, 1993; Whitehead & Douglas, 1993).

Although only bacteria morphologically similar to the ectosymbionts have been found in the cytopharynx and food vacuole of microzooids (Bauer-Nebelsick *et al.* 1996b), the host's diet is also known to depend on free-living bacteria from the water column (Rinke C., 2002). While colonies treated under OPNA conditions arose under the presence of microbes other than their ectosymbionts, colonies treated under OPA conditions arose under axenic conditions and the only microbes present were their own ectosymbionts. However, *Cand.* Thiobios zoothamnicoli had similar chemical properties related to growth and reproduction under both conditions.

The fitness of the corresponding colonies was estimated by counting the number of branches, which reflects the division rate of the terminal zooid (Rinke *et al.* 2007), after the seventh days of the experiment. A significantly lower fitness was estimated for colonies treated under OPA conditions (13.67 $\pm$ 5.18; N = 15) compared to those treated under OPNA conditions (24.61 $\pm$ 9.06; N = 18), which resulted in a much smaller colony size under OPA conditions than under OPNA conditions (pers. data. Volland J.-M., Kolar I., Drexel J. & Espada Hinojosa S., 2012).

Observed under the scanning electron microscope, colonies from both artificial treatments were seen to be covered with a monolayer of their ectosymbiont, with no difference between

the two artificial treatments in the number of symbionts, number of dividing cells per 70 µm² or the percentage of host surface coverage. This indicates that the size of the colonies in the ectosymbiont populations treated under OPA conditions was approx. 50% lower than those treated under OPNA conditions.

However, the symbionts had equal chemical conditions for growth and reproduction and their division rate was not significantly different between the two treatments, the host did not get overgrown by it´s symbionts under OPA conditions. This stays in contrast to observations made from other experiments: When *Z. niveum* samples were kept under optimal sulphide conditions and the a host died, it was sometimes see to be overgrown by its ectosymbionts (pers. observ.). This leads to the assumption, that a mechanism controlling the ectosymbiont population size has to be present when the host is alive.

In can be seen in literature that the bigger partner of a symbiotic association usually has a lower growth rate and controls the abundance and distribution of its partner by limiting the space it can occupy as well as its growth and proliferation (see Douglas, 2010). Control through space restrictions is seen with South American *Acacia*, which controls the number of symbiotic ants through the amount and size of the hollow thorns made available for the ants to live in (see Douglas, 2010). The space *Cand.* Thiobios zoothamnicoli can cover is restricted by the size and therefore by the amount of surface of *Z. niveum* itself.

Focusing on the evaluated parameters, the differences between the ectosymbionts of the two conditions were seen to be morphological, with ectosymbionts from the OPA treatments significantly smaller and rod-shaped. Control of symbiont growth and division rate by the host has been noted multiple times in literature, for example the bacterium *Buchnera* in aphids (Whitehead & Douglas, 1993) and the *Blattabacterium* in cockroaches (Brooks & Richards, 1955). The fitness of the *Cand.* Thiobios zoothamnicoli populations of the OPNA and OPA treatment, however, was equally high.

The number of symbionts covering the host surface must be restricted by some parameter. It is possible that the symbionts left the association with its partner and/or were kicked out. An example of this is the symbiosis between the squid *Euprymna scolopes* and the luminescent bacterium *Vibrio fischeri*. During the night, this bacteria has a concentration of up to 10^{11} cells ml^{-1}, densely packed in the light organ of their host. At the break of dawn they are released

into the surrounding water, with only approximately 10 % of the symbionts retained to renew the symbiotic population for the following night (Ruby & Asato, 1993).

Another possibility is that the symbionts treated under OPA conditions were subject to a higher feeding pressure by the host, due to the absence of other, free-living microbes. The host was, in fact, seen to grow, which means it had to have fed on something. However, autoradiographic investigations with corresponding samples incubated with ^{14}C in a pulse/chase experiment would be required to examine the difference in feeding-pressure on the ectosymbionts under axenic and non-axenic conditions.

4.3 Metabolic diversity

An investigation of the symbiosis with fully grown *Z. niveum* colonies and an established symbiont population under sulphide starvation conditions for *Cand*. Thiobios zoothamnicoli found that this symbiosis was still maintained after 48 hours. A similar investigation showed that under unfavorable conditions for the symbiont, the symbiosis was maintained until the host died (pers. obs. Bright, M., 2013). Our investigation also found that while the number of cells significantly decreased, the remaining ectosymbionts were larger in volume and still demonstrated fitness. These findings led to the conclusion that *Cand.* Thiobios zoothamnicoli is capable of utilizing nutritional sources other than sulphide.

The number of cells and the percentage of host surface coverage declined significantly for both the FLOW and NO FLOW treatments as compared to the *in situ* situation. There are a number of possible reasons for the significant reduction in cell number: In addition to the potential higher feeding pressure, the ectosymbionts decline could be due to self-detachment by the ectosymbionts, exclusion of the ectosymbionts or simply a higher death rate due to unfavorable environmental conditions. Nevertheless, the magnitude of cell loss must be higher than the division rate as a decrease in cell number was observed.

The significant changes in morphology were observed for the remaining ectosymbionts compared to those of the *in situ* samples. A large increase in cell volume was found, with highest measured values for NO FLOW ectosymbionts. CI was also seen to greatly increase, deriving mainly from increased length, with highest measured values for the FLOW ectosymbionts. In addition to the more voluminous ectosymbionts found in both artificial treatments, some sam-

ples treated under FLOW conditions were found to be covered in bulging, multiple dividing filaments supposed to be *Cand.* Thiobios zoothamnicoli (see previous section).

Furthermore, the remaining ectosymbionts still showed fitness. The FDC was seen be similar for *in situ* and FLOW ectosymbionts. Ectosymbionts treated under NO FLOW conditions had a significantly lower FDC, although this value was comparable to those measured for the *in situ* samples from Calvi, Corsica (Rinke *et al.* 2007).

These findings suggest that the ectosymbionts were still active under both artificial treatments. As sulphide was not available in the two treatments, the question arose: What other substances can the ectosymbionts feed on?

Unfortunately no data were available for those *Z. niveum* samples which were white or translucent when collected after 48 hours of experiment as these samples would have provided information on depletion of the ectosymbiont's elemental sulfur storage. However, it is known that elemental sulfur storage lasts for 4 hours when the ectosymbionts have no access to sulphide (Ott *et al.* 1998).

The potential for thiotrophic bacteria to use thiosulfate as an energy source has already been proven for the Alpha-Proteobacteria *Paracoccus*, which uses the Sox-gene cluster, and the Archaeon *Acidianus ambivalens* of the order sulfolobales, which generates a H^+-gradient and therefore ATP by enhancing a quinon-quinol pathway geared by thiosulfate oxydation (see Fuchs & Schlegel, 2006). Another bacterium which is able to feed autotrophically on thiosulfate is the facultative chemolithoorganotroph bacterium *Thiomicrospira thyasirae* (as mentioned above) (Wood & Kelly, 1993). Observations with *Z. niveum* and *Cand.* Thiobios zoothamnicoli treated with 1mM thiosulfate showed that the colonies died after 15 to 27 hours, while the ectosymbionts remained white as they did not deplete their elemental sulfur storage, left behind as white cloud covering the dead host (Rinke, 2002). The amount of thiosulfate in the water inflow of the FLOW experiment is not known, however it can be assumed that at least under NO FLOW conditions the amount of thiosulfate is low.

Another possibility is that Cand. Thiobios zoothamnicoli switched to a heterotrophic metabolism. The change from chemolithoautotrophy to heterotrophy has already been implied for the gammaproteobacterium *Cand.* Endoriftia persephone, the endosymbiont of *Riftia pachyptila,* by metagenomic analysis. In additIon to the endosymbiotic state, *Cand.* Endoriftia perse-

phone exists as free-living, where it is thought to favor the heterotrophic life-style, which may also explain why it can appear at sites where no sulphide is available (Robidart *et al.* 2008). Furthermore, it is known that all autotrophic microorganisms are also able to use organic compounds as a carbon source and it is assumed that mixotrophy may be the norm in nature (see Karl, 1995). Metagenomic and functional investigations need to be conducted for *Cand.* Thiobios zoothamnicoli in order to determine if this bacterium is capable of switching to a heterotrophic life-style, which would explain how it shows such significant increases in growth and still divides in situations where no sulphide is available.

4.4 Aposymbiotic host

Where swarmers were used to establish a colony under sulphide starvation conditions for the symbiont, established host colonies with no symbiont attached were observed after 7 days. These findings constitute the first time aposymbiotic colonies could be established under artificial conditions.

Preliminary studies have already been conducted with aposymbiotic colonies and colonies raised under sulphide starvation conditions (see Ott *et al.* 2004; Rinke *et al.* 2007). However, here we provide the first evidence that aposymbiotic colonies may be established under artificial conditions.

A macrozooid 150 µm in diameter is covered with approximately 90,000 ectosymbionts (Bauer-Nebelsick *et al.* 1996a). It is not known if the swarmers from which the observed colonies arose were aposymbiotic. If not, the ectosymbionts colonizing the swarmer would have undergone the same fate as those ectosymbionts treated under FLOW and NO FLOW conditions, with the loss of ectosymbionts predominating the division rate (see previous section). The fact that these colonies became aposymbiotic may then derive from a third process: the dilution of the ectosymbiotic community by growing host tissue. This was not the case for the already fully grown colonies treated under FLOW and NO FLOW conditions.

The establishment of aposymbiotic colonies under artificial conditions presented here offers new research possibilities, such as determining the benefits gained through *Cand.* Thiobios zoothamnicoli by comparing aposymbiotic colonies with symbiotic ones. Aposymbiotic colonies have been associated with a much lower growth rate compared to colonies contain-

ing ectosymbionts (see Ott *et al.* 2004). In this context it is interesting to note, that measurements of the fitness of these *Z. niveum* colonies treated under NONA conditions were shown to be lower than those treated under OPNA and OPA conditions (pers. data. Volland J.-M., Kolar I., Drexel J. & Espada Hinojosa S., 2012). Furthermore, cultivations of Z. *niveum* under normoxic conditions have been carried out (Rinke *et al.* 2007), where the mean measured life span of 4.5 $\pm$ 1.2 days (mean $\pm$s.d.; N = 12) was significantly lower than for those *Z. niveum* colonies treated with low (between 33 and 3 µmol/L) and medium (mean $\pm$ s.d. = 59.57 + 2.53) sulphide concentrations. However, that study did not investigate if the cultured specimens under normoxic conditions were aposymbiotic.

Furthermore, the establisment of an aposymbiotic host offers the possibility to address questions such as: Is the host able to fulfill its life-cycle in the absence of its symbiotic partner? Can the symbiont be reinfacted again?

It would greatly improve our knowledge of this symbiosis if an aposymbiotic *Z. niveum* colony was able to reproduce and therefore fulfill its own life-cycle. Although *Z. niveum* had access to sulphide during the time of settlement in the chamber, swarmers can also settle at locations where no sulphide is available (pers. obs). It is currently thought that *Z. niveum* is in an obligatory symbiosis with its ectosymbiont (Bauer-Nebelsick *et al.* 1996a,b). Although the *Z. niveum* colonies are aposymbiotic, it is not known if these colonies can fulfill their life-cycle without the energy acquired from its ectosymbionts.

The aspect of reinfection by *Cand*. Thiobios zoothamnicoli or other bacteria is also of great interest. Lucinid clams *Codakia orbiculata* were kept under sulphide starvation for three to six months (Gros *et al.* 2012) or 5 months (Elisabeth *et al.* 2012) and successfully acquired their sulphide-oxidising endosymbionts when reintroduced into their natural habitat where a free-living community of this symbiont was present. *Codakia orbiculata* obtains its symbionts through horizontal transmission every generation anew and apparently retains this ability to acquire symbionts over its entire life-span (Gros *et al.* 2012; Elisabeth *et al.* 2012). However, in contrast to *Codakia orbiculata,* there is no information on free-living communities of *Cand.* Thiobios zoothamnicoli and, furthermore, *Z. niveum* transmits its symbiont vertically to their offspring (Bauer-Nebelsick *et al.* 1996a). However, the knowledge, if a free-living *Cand.* Thiobios zoothamnicoli population exists, and if *Z. niveum* is also capable to take up ectosym-

bionts horizontally, is of great interest. The aposymbiotic host itself can serve as a good research tool to get answers for this questions by exposing it to seawater from its natural habitat and or by placing a symbiotic host nearby and observe, if the aposymbiotic host gets reinfected.

5 CONCLUSION

Z. niveum symbiosis has been previously cultivated, with differences in *Cand.* Thiobios zoothamnicolis morphology and fitness between the *in situ* situation and under optimal non-axenic culture conditions observed (Rinke *et al.* 2007). This approach was again followed in this study, with *in situ* and optimal *in vivo* samples also investigated. In addition, an insight into how the symbiosis responds to unfavorable conditions was sought: Therefore, the response of *Cand.* Thiobios zoothamnicoli to unfavorable conditions, either though sulphide exclusion or restrictions for the host feeding on free-living microbes from the water column, was investigated.

The *in situ* samples and those specimens used for *in vivo* treatments were collected in a canal next to a saline in Piran, Slovenia. *Zothamnium niveum* symbiosis was treated *in vivo* under different conditions for either 48 hours or seven days. The specimens treated under optimal *in vivo* conditions (OPNA) were raised from swarmers in a flow-through respirometer aquaria under sulphide supplementation in 32 µm filtered sea water for seven days. To investigate the response of *Cand.* Thiobios zoothamnicoli to its host starvation, specimens were raised from swarmers in flow-through respirometer aquaria for seven days with sulphide supplementation and 0.2 µm filtered sea water (OPA). To get an insight into what happens when *Cand.* Thiobios zoothamnicoli is kept under sulphide starvation, two sets of fully grown colonies with ectosymbionts attached were placed for 48 hours either in a respirometer flow-through aquarium with a flow rate of approximately 50 ml/h of 32 µm filtered sea water and no sulphide supplementation (FLOW) or in a embryo dish filled with 0.2 µm filtered sea water (NO FLOW). Furthermore, colonies were raised from swarmers in flow-through respirometer aquaria with a flow rate of approximately 50 ml/h of 32 µm filtered sea water and no sulphide supplementation for seven days (NONA).

All received specimens were prepared for and scanned with a scanning electron microscope (SEM) and several parameters related to morphology and fitness of the symbionts covering the hosts feeding cells were evaluated and compared.

When our *in situ* and the OPNA treatment were compared with each other as well as with *in situ* and optimal *in vivo* samples from sea-grass leaves in Calvi, Corsica in France (Rinke *et*

al. 2007), all four sample sets showed equal trends in the distribution of morphology and fitness parameters from the oral to the aboral part of the microzooid. However, differences appeared when the actual mean values were compared. These findings were interpreted as a response to the actual chemical conditions to which the *Z. niveum* symbiosis was exposed. This work further supports the classification of *Cand.* Thiobios zoothamnicoli as a pleomorphic cell-type (Bauer-Nebelsick *et al.* 1996a,b; Rinke *et al.* 2006). In addition, *Cand.* Thiobios zoothamnicoli was seen to undergo extreme morphological changes under sulfide starvation and it was therefore supposed that this cell type is capable of forming the bulging filaments observed. The substance from which these bulges were composed could not be identified in our study, but it would be interesting to know they comprised a dispersal unit of *Cand.* Thiobios zoothamnicoli built under unfavorable conditions. Investigations with DAPI or SYBR-green stainingmay serve as a suitable method to test this proposition.

It is known that a host´s diet relies partly on its symbiont, through digestion of carbohydrates excreted by symbionts after fixation and by direct feeding on the symbionts (Rinke, C. 2002). To get an insight into what happens when the host's normal food composition is restricted, the OPNA and OPA treatments were compared. The hosts showed a much lower proliferation rate where there was no access to free-living microbes. In contrast, sulfide was available in both conditions and the *Cand.* Thiobios zoothamnicoli populations were seen to have equal fitness. However, the symbiont did not overgrow the smaller hosts grown under unfavorable conditions, which is in contrast to observed overgrown dead hosts in other experiments (pers. observ.). It was therefore concluded that the factor controlling the homeostasis between the two partners of this symbiosis stems from the host, as supported by other examples of symbiosis in literature (see Douglas, 2010; Brooks & Richards, 1955; Ruby and Asato, 1993; Whitehead & Douglas, 1993).

In addition to the analysis of unfavorable conditions for the host, the symbiosis was also investigated under unfavorable conditions for *Cand*. Thiobios zoothamnicoli, with specimens kept under sulfide starvation. The symbiosis was seen to be still maintained on fully grown *Z. niveum* colonies treated under FLOW and NO FLOW conditions for 48 hours. While the number of ectosymbionts significantly decreased, the remaining cells were larger in volume and still demonstrated division. A similar experiment has shown that the symbiosis is still maintained until the host dies (pers. obs. Bright M., 2013). These findings led to the conclusion

that *Cand.* Thiobios zoothamnicoli is capable of using nutritional sources other than sulfide. As proposed for *Cand.* Endoriftia persephone (Robidart *et al.* 2008), we suspect that *Cand.* Thiobios zoothamnicoli is able to change from chemolithoautotrophy to heterotrophy. This is further supported by the fact that all autotrophic microorganisms can use organic compounds as carbon source and it is thought that mixotrophy may be the norm in nature (see Karl, 1995). However, metagenomic and functional investigations still need to be conducted for *Cand.* Thiobios zoothamnicoli in order to determine if this bacterium is capable of living heterotrophically.

In contrast to the maintained symbiosis of fully grown colonies with an established ectosymbiont population, *Z. niveum* samples which arose from swarmers under sulfide starvation conditions for seven days (NONA) were no longer seen to be in an association with their ectosymbionts. These findings constitute the first time aposymbiotic colonies could be established under artificial conditions. It is suspected that the symbionts were lost due to dilution through the growth of the colonies. This would be an additionally factor to those thought to act on fully grown colonies *Z. niveum* symbiosis treated under sulfide starvation: a potential higher feeding pressure, self-detachment by the ectosymbionts, exclusion of the ectosymbionts by the host or simply a higher death rate due to unfavorable environmental conditions. However, the fate of the ectosymbionts could not be determined, with this question still to be answered. Further questions arising from this work relate to the ability of an aposymbiotic host to fulfill its own life-cycle and if an aposymbiotic host can be reinfected. The transmission of *Cand.* Thiobios zoothamnicoli is known to operate vertically (Bauer-Nebelsick *et al.* 1996a) and in fact, there is no information available on free-living populations of *Cand.* Thiobios zoothamnicoli. It can be seen that there are many of aspects concerning this symbiosis still unknown, however, the establishment of aposymbiotic colonies is a promising tool with which to address these issues.

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7 APPENDIX

7.1 Chemical conditions

Measured Parameters		IN VIVO – optimal sulfidic, non-axenic conditions under flow, 7 days	IN VIVO – optimal sulfidic, axenic conditions under flow, 7 days		IN VIVO – normoxic, non-axenic conditions under flow, 2 days	IN VIVO – normoxic, non-axenic conditions under flow, 7 days		
		AQUARIA N°1	AQUARIA N°1	AQUARIA N°2	AQUARIA N°1	AQUARIA N°1	AQUARIA N°2	AQUARIA N°3
Oxygen concentration [µmol/L]	Mean value	254.03	264.33	264.19	261.27	277.64	276.29	276.11
	Standard diviation	16.77	6.48	12.49	7.72	7.12	9.30	6.45
	Maximum value	286.70	273.00	280.00	267.90	286.80	288.30	286.60
	Minimum value	225.00	254.30	246.30	252.80	268.90	262.70	269.10
	N° of measured values	14	11	7	4	12	12	10
Sulfide concnetration [µmol/L]	Mean value	16.33	21.90	18.98	n.d.	n.d.	n.d.	n.d.
	Standard diviation	21.94	9.35	9.48	n.d.	n.d.	n.d.	n.d.
	Maximum value	63.96	34.15	37.29	n.d.	n.d.	n.d.	n.d.
	Minimum value	-6.12	4.60	2.25	n.d.	n.d.	n.d.	n.d.
	N° of measured values	14	12	11	4	12	12	10
pH	Mean value	8.14	8.14	8.14	8.08	8.06	8.06	8.09
	Standard diviation	0.03	0.03	0.03	0.05	0.09	0.09	0.04
	Maximum value	8.19	8.19	8.18	8.14	8.17	8.13	8.15
	Minimum value	8.08	8.09	8.10	8.03	7.81	7.80	8.05
	N° of measured values	14	12	11	4	12	12	10
Temperature [°C]	Mean value	21.96	22.74	22.80	22.55	22.05	22.04	22.24
	Standard diviation	0.79	0.86	0.88	0.87	0.84	0.84	0.77
	Maximum value	23.30	24.00	24.00	23.30	23.30	23.30	23.30
	Minimum value	20.80	21.10	21.10	21.30	20.80	20.80	21.30
	N° of measured values	14	12	11	4	12	12	10
Salt concentration [‰]	Mean value	33.57	33.46	33.45	34.50	33.96	34.21	34.20
	Standard diviation	0.27	0.26	0.15	0.58	0.26	0.33	0.26
	Maximum value	34.00	34.00	33.50	35.00	34.50	34.50	34.50
	Minimum value	33.00	33.00	33.00	34.00	33.50	33.50	34.00
	N° of measured values	14	12	11	4	12	12	10
Flow rate [ml/h]	Mean value	48.77	48.52	45.87	50.00	49.17	48.21	51.00
	Standard diviation	2.80	3.46	4.65	2.83	1.75	4.37	3.53
	Maximum value	54.00	52.00	50.00	54.00	52.00	60.00	60.00
	Minimum value	44.00	40.00	35.00	48.00	47.00	44.00	47.00
	N° of measured values	14	12	11	4	12	12	10

7.2 Measured parameters

7.2.1 Within all treatments – oral vs. aboral part of the microzooid within one treatment

	IN SITU		IN VIVO – optimal sulfidic, non-axenic conditions under flow, 7 days		IN VIVO – optimal sulfidic, axenic conditions under flow, 7 days		IN VIVO – normoxic, non-axenic conditions under flow, 2 days		IN VIVO – stagnant, normoxic conditions, 2 days		IN VIVO – normoxic, non-axenic conditions under flow, 7 days	
Measured Parameters	Oral	Aboral	Oral	Aboral	Oral	Aboral	Oral	Aboral	Oral	Aboral	Oral	Aboral
cell elongation index	-	+	-	+	-	+	-	+	-	+	n.d.	n.d.
Volume [µm³]	+	-	+	-	+	-	+	-	+	-	n.d.	n.d.
FDC [%]	+	-	+	-	=	=	=	=	=	=	n.d.	n.d.
N° of dividing cells / 70 µm²	-	+	=	=	=	=	-	+	-	+	n.d.	n.d.
% of host surface coverage	=	=	=	=	=	=	-	+	-	+	n.d.	n.d.

(+ signifcantly higher; - significantly lower; = no significant difference)

7.2.2 Between all treatments – oral part of the microzooid

Measured Parameters		IN SITU	IN VIVO – optimal sulfidic, non-axenic conditions under flow, 7 days	IN VIVO – optimal sulfidic, axenic conditions under flow, 7 days	IN VIVO – normoxic, non-axenic conditions under flow, 2 days	IN VIVO – stagnant, normoxic conditions, 2 days	IN VIVO – normoxic, non-axenic conditions under flow, 7 days
		Part of the Microzooid	Part of the Microzooid	Part of the Microzooid	Part of the Microzooid	Part of the Microzooid	Part of the Microzooid
		ORAL	ORAL	ORAL	ORAL	ORAL	ORAL
length [µm]	Mean value	1.81	2.10	1.77	2.65	2.41	n.d.
	Standard deviation	0.46	0.52	0.46	0.94	0.62	n.d.
	Significance value	a	b	a	c	d	e
	N° of measured values	3150	2856	2608	1538	1841	
width [µm]	Mean value	0.87	0.86	0.69	0.78	0.94	n.d.
	Standard deviation	0.17	0.17	0.24	0.20	0.26	n.d.
	Significance value	a	a	b	c	d	e
	N° of measured values	3150	2856	2608	1538	1841	
volume [µm²]	Mean value	2.60	2.92	1.82	2.99	4.13	n.d.
	Standard deviation	1.27	1.41	1.68	2.13	2.70	n.d.
	Significance value	a	b	c	b	d	e
	N° of measured values	3150	2856	2608	1538	1841	
Cell elongation index	Mean value	2.16	2.53	2.77	3.60	2.79	n.d.
	Standard deviation	0.68	0.83	0.96	1.59	1.12	n.d.
	Significance value	a	b	c	d	c	e
	N° of measured values	3150	2856	2608	1538	1841	
N° of cells	Mean value	48.62	37.07	47.31	17.78	9.13	n.d.
	Standard deviation	10.42	7.22	17.16	9.43	12.10	n.d.
	Significance value	a	b	a	c	d	e
	N° of measured values	45	45	45	45	45	
Frequency of dividing cells [%]	Mean value	14.67	14.77	12.86	11.87	7.71	n.d.
	Standard deviation	2.12	2.79	2.85	3.40	3.57	n.d.
	Significance value	a	b	ab	c	d	e
	N° of measured values	45	45	45	45	45	
Absolute N° of dividing cells [N° / 70µm²]	Mean value	7.16	5.45	6.08	2.20	0.79	n.d.
	Standard deviation	1.92	1.48	2.67	1.60	1.11	n.d.
	Significance value	a	a	ab	b	c	d
	N° of measured values	45	45	45	45	45	
host surface coverage [%]	Mean value	88.16	87.01	83.51	51.47	22.24	n.d.
	Standard deviation	3.10	6.94	10.31	19.61	24.54	n.d.
	Significance value	a	a	a	b	c	d
	N° of measured values	45	45	45	45	45	

7.2.3 Between all treatments – aboral part of the microzooid

Measured Parameters		IN SITU	IN VIVO – optimal sulfidic, non-axenic conditions under flow, 7 days	IN VIVO – optimal sulfidic, axenic conditions under flow, 7 days	IN VIVO – normoxic, non-axenic conditions under flow, 2 days	IN VIVO – stagnant, normoxic conditions, 2 days	IN VIVO – normoxic, non-axenic conditions under flow, 7 days
		Part of the Microzooid	Part of the Microzooid	Part of the Microzooid	Part of the Microzooid	Part of the Microzooid	Part of the Microzooid
		ABORAL	ABORAL	ABORAL	ABORAL	ABORAL	ABORAL
length [µm]	Mean value	1.65	1.93	1.65	2.53	2.53	n.d.
	Standard deviation	0.44	0.50	0.42	1.13	0.72	n.d.
	Significance value	a	b	a	c	c	d
	N° of measured values	3088	2743	2324	1535	1489	
width [µm]	Mean value	0.59	0.72	0.60	0.64	0.75	n.d.
	Standard deviation	0.14	0.15	0.19	0.13	0.19	n.d.
	Significance value	a	b	a	c	d	e
	N° of measured values	3088	2743	2324	1535	1489	
volume [µm²]	Mean value	1.09	1.83	1.21	1.84	2.73	n.d.
	Standard deviation	0.75	0.92	1.00	0.99	1.78	n.d.
	Significance value	a	b	c	b	d	e
	N° of measured values	3088	2743	2324	1535	1489	
Cell elongation index	Mean value	2.93	2.79	2.93	4.10	3.54	n.d.
	Standard deviation	0.92	0.94	1.00	2.04	1.24	n.d.
	Significance value	a	b	a	c	d	e
	N° of measured values	3088	2743	2324	1535	1489	
N° of cells	Mean value	74.11	51.48	56.30	26.64	24.46	n.d.
	Standard deviation	14.95	11.76	18.07	8.46	12.35	n.d.
	Significance value	a	b	b	c	c	d
	N° of measured values	45	45	45	45	45	
Frequency of dividing cells [%]	Mean value	11.78	11.08	11.70	12.60	9.96	n.d.
	Standard deviation	1.93	2.30	1.90	3.21	3.75	n.d.
	Significance value	a	b	b	c	c	d
	N° of measured values	45	45	45	45	45	
Absolute N° of dividing cells [N° / 70µm²]	Mean value	8.68	5.65	6.53	3.50	2.60	n.d.
	Standard deviation	2.15	1.61	2.31	1.65	2.03	n.d.
	Significance value	a	a	a	ab	ac	d
	N° of measured values	45	45	45	45	45	
host surface coverage [%]	Mean value	89.80	89.30	84.85	64.02	64.36	n.d.
	Standard deviation	2.89	6.35	11.41	12.70	15.84	n.d.
	Significance value	a	a	a	b	b	c
	N° of measured values	45	45	45	45	45	

7.2.4 Between all treatments – total microzooid

Measured Parameters		IN SITU	IN VIVO – optimal sulfidic, non-axenic conditions under flow, 7 days	IN VIVO – optimal sulfidic, axenic conditions under flow, 7 days	IN VIVO – normoxic, non-axenic conditions under flow, 2 days	IN VIVO – stagnant, normoxic conditions, 2 days	IN VIVO – normoxic, non-axenic conditions under flow, 7 days
		Total Microzooid	Total Microzooid	Total Microzooid	Total Microzooid	Total Microzooid	Total Microzooid
length [µm]	Mean value	1.73	2.01	1.71	2.59	2.47	n.d.
	Standard deviation	0.45	0.52	0.44	1.03	0.68	n.d.
	Significance value	a	b	a	c	d	e
	N° of measured values	6238	5589	4932	3376	3027	
width [µm]	Mean value	0.73	0.79	0.65	0.72	0.85	n.d.
	Standard deviation	0.21	0.17	0.22	0.18	0.25	n.d.
	Significance value	a	b	c	a	d	e
	N° of measured values	6238	5589	4932	3376	3027	
volume [µm²]	Mean value	1.85	2.38	1.53	2.47	3.44	n.d.
	Standard deviation	1.29	1.31	1.43	1.80	2.40	n.d.
	Significance value	a	b	c	b	d	e
	N° of measured values	6238	5589	4932	3376	3027	
Cell elongation index	Mean value	2.54	2.66	2.84	3.83	3.16	n.d.
	Standard deviation	0.90	0.90	0.98	1.83	1.24	n.d.
	Significance value	a	b	c	d	e	f
	N° of measured values	6238	5589	4932	3376	3027	
N° of cells	Mean value	61.37	44.28	51.70	22.22	16.78	n.d.
	Standard deviation	18.12	12.11	18.07	9.97	14.38	n.d.
	Significance value	a	b	b	c	c	d
	N° of measured values	90	90	90	90	90	
Frequency of dividing cells [%]	Mean value	13.22	12.93	12.28	12.23	8.84	n.d.
	Standard deviation	2.48	3.15	2.48	3.31	3.81	n.d.
	Significance value	a	a	a	a	b	c
	N° of measured values	90	90	90	90	90	
Absolute N° of dividing cells [N° / 70µm²]	Mean value	7.92	5.55	6.30	2.85	1.70	n.d.
	Standard deviation	2.17	1.54	2.50	1.74	1.86	n.d.
	Significance value	a	b	b	c	d	e
	N° of measured values	90	90	90	90	90	
host surface coverage [%]	Mean value	88.98	88.15	84.18	57.75	43.30	n.d.
	Standard deviation	3.09	6.72	10.83	17.60	29.50	n.d.
	Significance value	a	ab	b	c	d	e
	N° of measured values	90	90	90	90	90	

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9 CURRICULUM VITAE

1997 -2005

BRG Dornbirn Schoren, Vorarlberg; A-levels.

2005 -

Masters in Ecology with main focus on Marine Biology at the University of Vienna.

2012

Diploma thesis in the field of the thiotrophic ciliate mutualism between *Cand.* Thiobios zoothamnicoli and *Zoothamnium niveum.*

Skills:

- Languages: German, English.
- Tutoress for a Projectpractica at the Biophysical Chemistry Institut at the University of Vienna in the laboratory of Prof. Dr. Georg Schmetterer - dealing with basic molecular techniques to investigate the molecular biology of cyanobacterias - in summer- and wintersemester 2011.