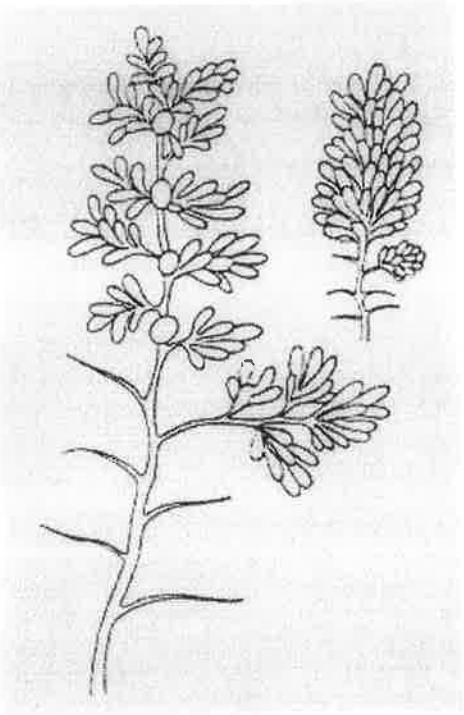
Carbonfixation -incorporation and -transfer in the chemoautotrophic *Zoothamnium niveum* symbioses with ^{14}C Bicarbonat Autoradiography

Kohlenstofffixierung, -inkorporation und -transfer in der chemoautotrophen *Zoothamnium niveum* Symbiose mit ^{14}C Bikarbonat Autoradiographie



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EINLEITUNG

Durch die Entwicklung neuer Techniken und durch die steigende Akzeptanz des symbiontischen Ursprungs der eukaryotischen Zelle gewann die Symbioseforschung in den letzten Jahrzehnten zunehmend an Bedeutung. Eine der faszinierendsten Aufgaben dieses Forschungsfeldes ist es, komplexe Beziehungen zwischen symbiontischen Bakterien und deren Wirtsorganismen zu charakterisieren. Im marinen Bereich finden sich, vor allem an der Grenze von oxidierenden und reduzierenden, schwefelhaltigen Zonen, einige der bemerkenswertesten Symbiosen. Entdeckt wurden diese Lebensgemeinschaften an den „hot vents" und „cold seeps" der Tiefsee (siehe Childress & Fisher 1992, Cavanaugh 1994, Nelson & Fisher 1995, Fisher 1996, Van Dover et al. 2002). Mittlerweile sind Beispiele aus allen marinen, sulfidischen Lebensräumen, wie brandungsgeschützte, organisch angereicherte Sedimente, Seegrasdebris und Mangroventorf bekannt. Die Lebensgemeinschaft basiert auf chemoautotrophen, schwefeloxidierenden Bakterien, welche in enger Assoziation mit Protisten (Fenchel & Finlay, 1989, Bauer-Nebelsick et al. 1996 a, b), Plathelminthen (Ott et al. 1982), Nematoden (Ott et al. 1982, Ott & Novak 1989), Polychaeten (Giere 1996), Oligochaeten (Giere 1981, Krieger et al. 2000, Dubilier et al. 2001), Echiuriden (Menon & Arp 1993), Priapuliden (Oeschger & Schmal-Johann 1988, Oeschger & Janssen 1991), Bivalven (Cavanaugh 1985, Felbeck et al. 1981), Gastropoden (Stein et al. 1988), Arthropoden (Gebruk et al. 1993, Polz et al. 1999), Echinodermen (Tamara et al. 1993) und Pogonophoren (Southward et al. 1986) leben.

Energiegewinn erfolgt bei den ekto- oder endosymbiontischen Bakterien durch die Oxidation reduzierter Schwefelverbindungen, wobei Sauerstoff als terminaler Elektronenakzeptor benutzt wird. Diese chemoautotrophen Symbionten ermöglichen die Ausbildung eines vom Sonnenlicht unabhängigen Nahrungsnetzes.

In den folgenden fünf Tierstämmen haben sich thiotrophe Ektosymbiosen entwickelt: Ciliophoren, Nematoden, Anneliden, Priapuliden und Arthropoden. Für Arthropoden, Nematoden und Ciliophoren sind zusätzlich Nahrungsbeziehungen beschrieben. Als Beispiele sind der Shrimp *Rimicaris ssp.* zu nennen, der Hydrothermalquellen in der Tiefsee bewohnt (Gebruk et al. 1993, Polz et al. 1999) sowie die Vertreter der Nematoden-Unterfamilie *Stilbonematinae*, die interstitiell in marinen Sedimenten anzutreffen sind (Polz et al. 1994). Auf Mangroventorf und verrottendem Pflanzenmaterial findet man die peritrichen marinen Ciliaten *Zoothamnium niveum* (Bauer-Nebelsick et al. 1996 a, Ott et al. 1998) und *Vorticella sp.* (eigene Beobachtungen), während der bandförmige und mundlose Ciliat *Kentrophorus sp.* (Finlay & Fenchel 1989, Foissner 1995) zwischen Sandpartikeln mariner Sedimente lebt. Stabile Isotopen Messungen zeigen, dass *Rimicaris spp.*, sowie die Nematoden der Unterfamilie *Stilbonematinae* auf sulfidoxidierende Epibionten als Nährstofflieferanten angewiesen sind (Ott et al. 1991). *Kentrophorus sp.* stülpt einen Teil seiner bakterienbesetzten Zelloberfläche ein und transferiert dadurch die Ektosymbionten zur Verdauung in Nahrungsvakuolen (Finlay & Fenchel 1989).

Für die Ektosymbiosen der Anneliden und Priapuliden werden nach dem derzeitigen Stand der Wissenschaft Nahrungsbeziehungen ausgeschlossen. Der bakterielle Aufwuchs des Priapuliden *Halicryptus spinulosus* soll für die Sulfidentgiftung dieser mutualistischen Beziehung verantwortlich sein (Oeschger & Janssen 1991). Die Aufwuchsgemeinschaft des Tiefsee-Polychaeten *Alvinella pompejana* ist sehr divers. Die häufigsten Bakterien sind zwei filamentöse Phylotypen, die für den Wirt nicht obligat erforderlich zu sein scheinen.

Die Art *Zoothamnium niveum* wurde vor 170 Jahren beschrieben. Die Autoren wählten aufgrund der weißen Farbe den Namen „niveum = verschneit", erkannten jedoch nicht, dass die Färbung durch einen bakteriellen Überzug der Oberfläche zustande kommt, der nur am basalen Teil des Stiels fehlt. Die Wiederbeschreibung von *Zoothamnium niveum* durch Bauer-Nebelsick (1996 a, b) zeigt, dass die federförmigen Kolonien aus einem Stamm bestehen, von

dem alternierend Äste ausgehen, die wiederum eine alternierende Anordnung von Zooiden aufweisen. Die Zellen dieses kolonialen Ciliaten können in drei Morphotypen mit unterschiedlichen Funktionen unterteilt werden. Mikrozooide besitzen eine gut ausgebildete Ziliatur mit der sie zur Filtrierung von Partikeln einen Wasserstrom erzeugen. Makrozooide lösen sich als sogenannte „Schwärmer" und bilden an geeigneten Standorten neue Kolonien aus. Diese großen runden Zellen verfügen über eine voll entwickelte Ziliatur. Ihr Zellschlund ist stark reduziert, wodurch das Ingestieren und Verdauen von Partikeln nicht möglich ist. Terminalzooide ermöglichen durch longitudinale Teilungen die Zellvermehrung und das Astwachstum (Bauer-Nebelsick et al. 1996 a). Die Kolonie ist von zwei morphologisch unterschiedlichen Bakterien bewachsen. Mikrozooide werden von kokkenförmigen Bakterien bedeckt, während Stiel, Äste und Makrozooide stäbchenförmige Symbionten tragen. Auf den Mikrozooiden sind zusätzlich bakterielle Zwischenformen zu finden. Dies führt zu der Vermutung, dass es sich um zwei Morphotypen einer bakteriellen Art handelt (Bauer-Nebelsick et al. 1996 a).

Farbe, Ultrastruktur und der Nachweis des CO_2 fixierenden Enzyms des Calvin-Benson-Zyklus, Ribulose-1,5-Bisphosphat Carboxylase/Oxygenase (RuBisCo) (H. Felbeck, pers. Mitt.), sowie Experimente mit Kartesischen Tauchern (Ott et al. 1998), weisen darauf hin, dass die Bakterien chemolithoautotroph sind. Kurzzeit-Kultivierungsexperimente in Sulfid und Thiosulfat zeigen, dass die Bakterien befähigt sind, beide Schwefelformen zu nutzen.

Der Lebensraum von *Zoothamnium niveum* ist die Mangroventorfwand in den Gezeitenkanälen karibischer Inseln. Messungen mit Mikrosensoren beweisen, dass die „Schwärmer" von *Z. niveum* Stellen mit Sulfidwerten bis 300 µM bevorzugen, um neue Kolonien zu bilden. Durch wiederholte Kontraktionen und Expansionen der gesamten Kolonie wechselt der Ciliat zwischen dem normal oxygenierten Seewasser und der nur wenige Millimeter hohen sulfidischen Grenzschicht am Mangroventorf (Ott et al. 1998). Die

Expansionen des Ciliaten bewirken den Transport von sulfidischem Seewasser aufwärts in die oxygenierte Zone (Vopel et al. 2001). Es wird vermutet, dass dieser advektive Flüssigkeitstransport, bedingt durch das Zucken der gesamten Kolonie, sowie durch die Bewegungen der oralen Ziliatur, die ektosymbiontischen Bakterien mit Sauerstoff und Sulfid versorgt (Vopel et al. 2001).

Die Nahrungsaufnahme erfolgt bei Ciliaten grundsätzlich durch Endocytose von Partikeln (Phagocytose) oder von Flüssigkeiten (Pinocytose). Gelöste Nährstoffe können direkt durch die Außenhülle (Cortex) aufgenommen werden (Osmotrophie). Der komplexe Cortex erlaubt Endo- und Exocytose nur an bestimmten permeablen Stellen der Plasmamembran, dem Cytosom (Zellmund) und dem Cytoproct (Zellafter). Der Großteil der Ciliaten ingestiert seine Nahrung über das Cytosom. Osmotrophie und Pinocytose treten nur gelegentlich auf (siehe Radek & Hausmann 1996).

Erste Hinweise einer nutritiven Beziehung in der *Zoothamnium niveum* Symbiose lieferten Beobachtungen an lebenden Individuen, die Bakterien ingestierten, welche in Größe und Ultrastruktur den Ektosymbionten ähnlich waren (Bauer-Nebelsick et al., 1996 b). Da ein direkter Beweis für eine Nahrungsbeziehung jedoch fehlt, gehen wir in dieser Studie der Frage nach, ob *Zoothamnium niveum* seine Ektosymbionten als Nahrung nutzt. Dies könnte entweder durch die direkte Aufnahme von niedermolekularen Stoffen, die von den Bakterien abgegeben werden, oder über das Einstrudeln und Ingestieren der Symbionten erfolgen. Außerdem interessieren wir uns für den möglicherweise auftretenden Kohlenstofftransfer zwischen den unterschiedlichen Zooidtypen.

Bei den Ektosymbionten untersuchen wir, ob sie bei unterschiedlichen chemischen Bedingungen Kohlenstoff fixieren und inkorporieren und ob es Unterschiede in der Inkorporation zwischen den beiden bakteriellen Morphotypen gibt. Um die Kohlenstoffinkorporation der Symbionten, die Verwertung der Bakterien bzw. ihrer metabolitischen Produkte durch den Wirtsorganismus und die Verteilung des inkorporierten

Kohlenstoffs in der Kolonie zu untersuchen, wird in dieser Studie die Technik der ^{14}C-Autoradiographie verwendet.

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ABSTRACT

The very large and fast growing sessile, colonial ciliate *Zoothamnium niveum* (Ciliophora, Oligohymenophora) inhabits peat surfaces of mangrove islands in the lagoonal regions of the Belize Barrier Reef. A coat of bacteria covers the host obligatorily. First indications of a nutritional relationship came from observations of live specimens that ingested bacteria identical to their own symbionts and from ultrathin sections with similar bacteria in food vacuoles in the process of degradation. The aims of this study were to investigate with ^{14}C bicarbonate tissue autoradiography under which conditions and time frames the autotrophic symbionts are capable of carbon fixation and incorporation and by which means transfer of fixed carbon from the symbionts to the host occurs. The results indicate that the symbionts are able to fix inorganic carbon and incorporate organic carbon incubated in normoxic, thiosulfatic, and sulfidic seawater. Incubations in seawater without added sulfur species are hypothesized to be due to the use of elemental sulfur stored in the symbionts and are in accordance with Cartesian diver experiments that demonstrated the oxidation of elemental sulfur within a few hours incubation time. The two bacterial morphotypes, cocci and rods were incorporating different amounts of organic carbon per cell. This difference is explained by the dissimilar sulfide and oxygen supply caused by behavior and morphology of the host. Transfer of fixed carbon from the symbionts to the host occurs via digestion of the symbionts and via uptake of low molecular release from the symbionts. While fast bacterial release is seen already after 15 min of pulse labeling, the slower digestional process is seen in the increase of label over host tissue between 3 hour pulse and 24 hour chase periods. Translocation of digested nutrients from the feeding microzooids to the non-feeding other parts of the colony, such as macrozooids, stalk, and branches occurred fast, since label over all parts of the host was detected simultaneously. This transfer is hypothesized to occur via cytoplasmic connections. Our experiments clearly point to a tight nutritional relationship

between a thiotrophic ectosymbiont and *Z. niveum*. The host is capable to uptake symbionts release of organic carbon and to ingest its own symbionts, but at the same time, most likely also consumes free-living bacteria from the surrounding water. The specific contribution of the symbionts to the host's diet still remains to be determined.

INTRODUCTION

Symbioses between chemolithoautotrophic, sulfur oxidizing bacteria and protists or invertebrates were first discovered at the deep sea hydrothermal vents on the Galapagos Rift in 1977 (Corlis et al. 1979). Up to now, these marine associations have been described from several hydrothermal vent sites and cold seeps (see Nelson and Fisher 1995, Fisher 1996) as well as from shallow water reducing habitats (see Ott 1996, Giere 1996, Bright 2002, Ott 2002). So far we know of five eukaryotic phyla, in which thiotrophic ectosymbiosis evolved, Ciliophora, Nematoda, Annelida, Priapulida, and Arthropoda (see Fisher 1996). For at least three host phyla, symbiosis with a considered nutritive relation are described. The shrimps *Rimicaris spp.* inhabitat hydrothermal vents (Gebruk et al. 1993, Polz et al. 1999), and members of the nematode subfamily *Stilbonematinae* grow interstitially in tropic and temperate shallow water sediments (Ott 1996, Polz et al. 2000). Among the protists the peritrich ciliates *Zoothamnium niveum* (Bauer-Nebelsick et al. 1996a) and *Vorticella sp.* (own observations) occur on mangrove peat, the ribbon-like ciliate *Kentrophorus sp.* (Fenchel and Finlay 1989) inhabits temperate marine sediments. Staple isotope measurements indicate that *Rimicaris spp.* (Polz et al. 1999) as well as representatives of the nematode subfamily *Stilbonematinae* rely on their sulfur oxidizing epibionts as a food source (Ott et al. 1991). The mouthless ciliate *Kentrophorus sp.* feeds on its attached bacteria by phagocytosis (Fenchel and Finlay 1989, Foissner 1995).

Zoothamnium niveum (Ciliophora, Oligohymenophora) is obligatorily covered with a white bacterial coat. The color of the bacteria, which is supposed to be due to elemental sulfur inclusions and the presence of the CO_2 fixing enzyme of the Calvin-Benson cycle, ribulose-1,5-bisphosphate carboxylase/oxygenase (H. Felbeck pers. comm.) as well as oxygen uptake experiments with cartesian divers (Ott et al. 1998) indicate that the ectosymbionts are sulfide-oxidizing chemolithoautotrophs. Short term cultivation experiments in sulfide or thiosulfate

added to seawater suggest that the bacteria are capable of oxidizing both sulfur species (own obs.), but so far it has not been clearly demonstrated .

The habitat of the symbioses is the mangrove peat walls along the banks of tidal channels and ponds in the Caribbean Sea. Measurements with microsensors showed that the dispersal stages of *Zoothamnium niveum* preferentially settle at the peat surface in areas with H_2S up to 300µM to build up a new colony. By repeated contractions and expansions, the host changes between anoxic, sulfidic seawater close to the peat surface and sulfide-free, normoxic seawater above (Ott et al. 1998). Through expansions sulfidic seawater is transported upwards into the oxygenated zone. It was suggested that this advective fluid transport, caused by the movement of the entire colony as well as the feeding current of the oral ciliature, supplies the epibiotic bacteria with oxygen and sulfide (Vopel et al. 2001).

The feather-like colony exhibits a central stalk, branches occurring alternately on the stalk, and three cell morphotypes on the branches: the microzooids are the feeding stages. The macrozooids are the dispersal stages. They are capable of leaving the colony as large swarmers and built a new colony after settlement. They possess a fully developed oral ciliature but only a reduced cytopharynx, thus ingestion and digestion was questioned. Terminal zooids are responsible for reproduction by longitudinal fission (Bauer-Nebelsick et al. 1996a, b). The symbiotic coat consists of two morphotypes: stalk, branches, terminal- and macrozooids are covered with rod-shaped symbionts, whereas microzooids serve as a substrate for slightly dumbbell-shaped coccoid microbes. A complete series of intermediate shapes between cocci on the oral side and rods on the aboral side of the microzooids suggest that the two morphotypes belong to the same bacterial species (Bauer-Nebelsick et al. 1996b).

In ciliates, food uptake occurs in general via endocytosis of particles (phagocytosis) or of fluid substances (pinocytosis), whereas dissolved nutrients are internalized by transmembrane transport (osmotrophy). The complex cortex of ciliates allows endo- and exocytosis only on distinct sites, the cytostome and the cytoproct. For most ciliates, the

ingestion via the cytostome plays the central role in the uptake of nutrients, osmotrophy and pinocytosis occur apparently (see Radek and Hausmann 1996).

The first indications of a nutritional relationship in the *Zoothamnium niveum* symbiosis came from observations of live specimens that ingested bacteria identical to their own symbionts and ultrathin sections with bacteria, similar in ultrastructure and in size to the symbionts, in food vacuoles in the process of degradation (Bauer-Nebelsick et al. 1996 b). However, since a direct evidence for a nutritive association did not exist, we aimed to examine whether or not *Z. niveum* feeds on the ectosymbiotic bacteria. Direct uptake of low molecular weight products, released directly from the bacteria (milking), or uptake of symbionts by ingestion and digestion (farming) could nourish the host. Additionally, we were interested in the may occurring carbon transfer between the different host morphotypes. Concerning the ectosymbionts, it was questioned whether or not the bacteria autotrophically fix and incorporate carbon under different chemical conditions, and whether or not a difference in carbon uptake occurs between the two different morphotypes.

For our approach, ^{14}C bicarbonate tissue autoradiography under different chemical conditions and time frames was applied in pulse-chase experiments to investigate the nutritive associations of the symbiosis, the possible transfer of organic carbon within the host colony, and the carbon uptake capability of the ectosymbionts. However, tissue autoradiography can only provide semiquantitative data, because many soluble products of carbon fixation are extracted during fixation and dehydrating procedures (Rogers 1979). Only incorporation into relatively insoluble, high molecular weight organic carbon can be seen as silver grains over specific tissues. Radioactive labeled low molecular weight carbon, which could be released from bacterial symbionts, will not be detected until incorporation into less soluble larger molecules in host tissues occurs.

MATERIALS AND METHODS

^{14}C Bicarbonate tissue autoradiography incubations. Specimens of *Zoothamnium niveum* were collected from the peat surface in the main channel of the mangrove island Twin Cays (Belize Barrier Reef, Caribbean Sea) in April, 2000. To avoid microbial contaminations the seawater was filtered with a 0.2 µm filter. Following incubations were carried out in 2.5 ml glass vials at 28°C (Table 1): 1) short pulse for 15 min containing 100 µC ml $^{-1}$ $NaH^{14}CO_3$ in 100 µM hydrogen sulfide (SU), 1 mM thiosulfate (TH), or normoxic seawater (SW); 2) long pulse for 3 hours containing 100 µC ml^{-1} $NaH^{14}CO_3$ in 100 µM hydrogen sulfide (SU), 1 mM thiosulfate (TH), or normoxic seawater (SW); 3) short chase (long pulse incubations in sulfide or thiosulfate followed by chase incubations in 1 mM thiosulfate (TH) without radioactive source, or long pulse incubations in normoxic seawater followed by chase incubations in normoxic seawater (SW) without radioactive source for 12 hours); 4) long chase (long pulse incubations in sulfide or thiosulfate followed by chase incubations in 1 mM thiosulfate (TH) without radioactive source, or long pulse incubations in normoxic seawater followed by chase incubations in normoxic seawater (SW) without radioactive source for 24 hours). The sulfide concentration decreased approximately 10 times within 3 hours, due to the fact that sulfide oxygenates to sulfate. Therefore, all chases except the normoxic seawater incubations were carried out in 1 mM thiosulfate. Control incubations without added ^{14}C bicarbonate were applied. For each treatment a minimum of 5 specimens were incubated.

After the applied incubations, the specimens were rinsed several times in filtered seawater and fixed in 4% paraformaldehyde in phosphate puffer, pH 7.2, 0.15 M (see Bright et al. 2000). After dehydration in a series of ethanol, the specimens were embedded in SPURR'S epoxy resin, longitudinally semithin sectioned (1 µm) on a Reichert Ultracut E and mounted on slides dipped in 0.5 % gelatine with 0.05% chromalaune. The slides were dipped in Kodak NTB2 emulsion in absolute darkness, allowed to dry and placed in light tight boxes

containing bags of desiccant, each slide facing the same direction and separated by one slot form the neighboring slide. As control for positive chemography, sections from control incubations were used. Negative chemography was tested with slides exposed to a light bulb for approximately 1 min. After an exposure time of 9 days at 4°C, the slides were developed with Kodak Developer D-19, fixated with Kodak Fixer according to the manufacturer's instructions, rinsed in distilled water, air dried and stained in 0.25% borax, 0.25% methylene blue, 0.25% toluidine blue, 0.25% azur II after Erickson et al. (1990).

Analysis and Statistical Methods. The total size of the colonies ranged between 0.6 to 1 cm. Only sections exhibiting microzooids, macrozooids and stalks were used. The silver grains over the tissue were observed, using a Reichert Polyvar light microscope, images were captured with a Philips digital camera and the software analySIS®. Digital images were printed on a laser printer. The silver grains of the following areas (10 x 20 µm) were counted: 1) host, 2) host and symbionts, 3) symbionts, and 4) background (Fig. 1) (to lighten the comprehensibility all 6 areas will be referred to as "tissue"). Within the colony we distinguished between three different areas comprising host tissue and adjacent symbionts each: microzooids with cocci; macrozooids with rods; and stalk with rods.

In order to test whether silver grains over host and symbiont are due to incorporation or unspecific label, the counted grains over the host and symbiont were tested against the background (post hoc Tamhane test, confidence level 99%). Only specimens in which the silver grain density over the symbiont tissue showed a significant difference to the background density were counted as autotrophically active and were used for further statistical analyses. When the silver grain density was not significant different between background and specific host tissues, indicating no incorporation, the specific host areas were taken as 0 and included in further statistical analyses. In order to avoid scattering and overlapping of radioactivity from the areas 'host' and 'symbionts', the area 'host and

symbionts' was left as space between the two partners, and therefore the silver grains over this area were not included in the statistical process.

In the positive chemography control with specimens that were incubated without radioactive source, no label was detected. In the negative chemography control, the light exposed slides showed a dense layer of silver grains, proving that the autoradiography procedure was working.

Comparisons between the actual grain densities AGD (i.e. the number of silver grains counted in a specific area) between different slides from the same specimen and between different specimens are inappropriate due to variations in section and emulsion thickness. To allow a semi-quantitative statistical comparison, the AGDs were transformed to relative grain densities (RGD) according to Rogers (1979). The AGDs over the symbionts covering the microzooids, the macrozooids, and the stalk were weighted to 33.3% each, added and set to 100 relative grain density units. All RGDs were calculated as percentage of the 100 relative grain density units (Table 2).

Non-parametric statistics were used to compare RGDs because the assumption of normality is often unjustified for grain densities over biological specimens (Rogers, 1979). The Kruskal-Wallis H-test with a 99% confidence interval was used to compare more than two data sets, such as the time frames (short pulse, long pulse, short chase, long chase), the different chemical conditions (100 μM hydrogen sulfide, 1 mM thiosulfate, normoxic seawater), and the six 'tissue' types (microzooid, macrozooid, stalk, cocci on microzooids, rods on macrozooids, and rods on stalks). In order to compare multiple data sets, the Tamhane post hoc multiple comparison test for unequal variances (SPSS®9.00) with a 99 % confidence interval was used.

Table 1. ^{14}C bicarbonate tissue autoradiography experiments. The incubations under different chemical conditions, normoxic seawater (SW), 1mM thiosulfate added to seawater (TH), and 100 µM ∑ sulfide added to seawater (SU) were carried out for the following times: short pulse (SP, 15 min), long pulse (LP, 3 hours), short chase (SC, 3 hours pulse + 12 hours chase), long chase (LC, 3 hours pulse + 24 hours chase). Number of specimens (n_1) that were incubated for each experiment (exp.), number of specimens (n_2) expressed as percentage of total specimens that survived the experiments, number of specimens (n_3) expressed as percentage of total specimens that were autotrophically active, and number of specimens (n_4) that were taken for statistical analyses (an.).

incubation time	**pulse incubation (100 µCi ml^{-1} $NaHCO_3$)**	**chase incubation (no radioactive source)**	**n_1 (exp.)**	**n_2 % (survival)**	**n_3 % (autotrophy)**	**n_4 (an.)**
short pulse (SP) 15 min	SW (normoxic)	-	7	100	86	3
	TH (1mM thiosulfate)	-	10	100	70	3
	SU (100 µM ∑ sulfide)	-	9	100	67	3
long pulse (LP) 3 hours	SW	-	6	100	83	3
	TH	-	10	100	80	4
	SU	-	9	100	78	4
short chase (SC) 3 hours + 12 hours	SW	SW	10	0	0	0
	TH	TH	5	100	100	4
	SU	TH	5	100	100	3
long chase (LC) 3 hours + 24 hours	SW	SW	10	0	0	0
	TH	TH	10	0	0	0
	SU	TH	6	100	83	5

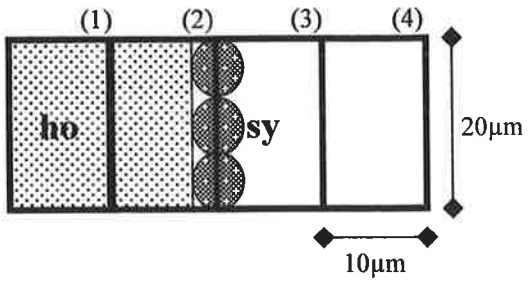

Fig. 1. Schematic drawing of the counting rectangle, containing 4 areas of 10 x 20 µm over host tissue (1), host and symbiont tissue (2), symbiont tissue (3), and the background (4), to count the silver grains on sections coated with autoradiography emulsion. The host (ho) is light stippled and the ectosymbionts (sy) are dark stippled in the drawing.

RESULTS

The survival of the *Zoothamnium niveum* symbiosis was strongly influenced by the incubation media, SW, TH, or SU and the incubation time. All hosts and symbionts survived incubations in SW, TH, and SU for 3 hours. By the end of these long pulse incubations, the hosts exhibited continuous contraction/expansion behavior and the oral cilia of the microzooids were actively beating after expansions. The symbionts remained white. All specimens incubated in SW died during longer incubations between 3 and 12 hours, while all specimens incubated in TH and SU survived this time period. During the long chase period between 15 and 27 hours, the hosts died but the symbionts stayed white in TH and both partners survived in SU. In all applied experiments 67% to 100% of the surviving specimens were autotrophically active (Table 1). The incubations in SU showed a lower percentage of autotrophically active symbionts than the incubations in SW and TH during the short and long pulse periods.

The RGDs over the symbiotic morphotypes, cocci on microzooids, rods on macrozooids, and rods on stalks were not significantly different in the three incubation media at any time frame each (post hoc Tamhane test, confidence level 99%). Therefore, the data of all three morphotypes were combined into 'symbionts' for further comparisons with host tissues. A comparison between symbionts and host showed that after the short pulse period, the RGDs over symbiotic tissue were significantly higher than over the host tissue in all three incubation media (post hoc Tamhane test, confidence level 99%) (Fig. 2a, 2b). After the long pulse period, the RGDs over symbiotic tissue remained higher than over the host tissue in SW, but were not significantly different between symbiotic and host tissue in each TH and SU (post hoc Tamhane test, confidence level 99%) (Fig. 2c, 2d). These similar levels were also found after the short chase period. Only after the long chase period in SU, the symbiotic tissue

was significantly less labeled than the host tissue (post hoc Tamhane test, confidence level 99%).

The incubation media had a strong impact on the incorporation rate of organic carbon in host tissue (Fig. 3, table 2). After the short pulse period, the RGDs over host tissue were significantly higher in SW and TH than in SU. In SW, the RGDs were highly variable (37.67 ± 33.82%) and were due to incorporation in microzooids and stalks, while the RGD of the macrozooids was not significantly different from the adjacent background. In TH, the RGD was due to incorporation in microzooids (27.71 ± 13.07), the macrozooids and the stalks showed no incorporation. In SU, no incorporation was detected in any host tissue. After the long pulse period, the RGDs over host tissue were significantly lower in SW than in TH or SU. Similar levels of incorporation were also noticed in TH and SU after the short chase period, while the symbiosis died in SW during that time.

The applied time frames also strongly influenced the label over host tissue (Fig. 4, table 2). In SW, the RGD of the host tissue decreased significantly while in TH and SU, the RGDs increased significantly between short and long pulse periods. The strong label over host tissue was 102.28 ± 92.15 % in TH and 125.92 ± 40.24% in SU after the long pulse period and remained similar after the short chase periods in both reduced sulfur incubations. Only after the long chase period in SU, the increase of RGD over host tissue to 240.27 ± 63.82 % became distinct resulting in a significant increase between long pulse and long chase periods.

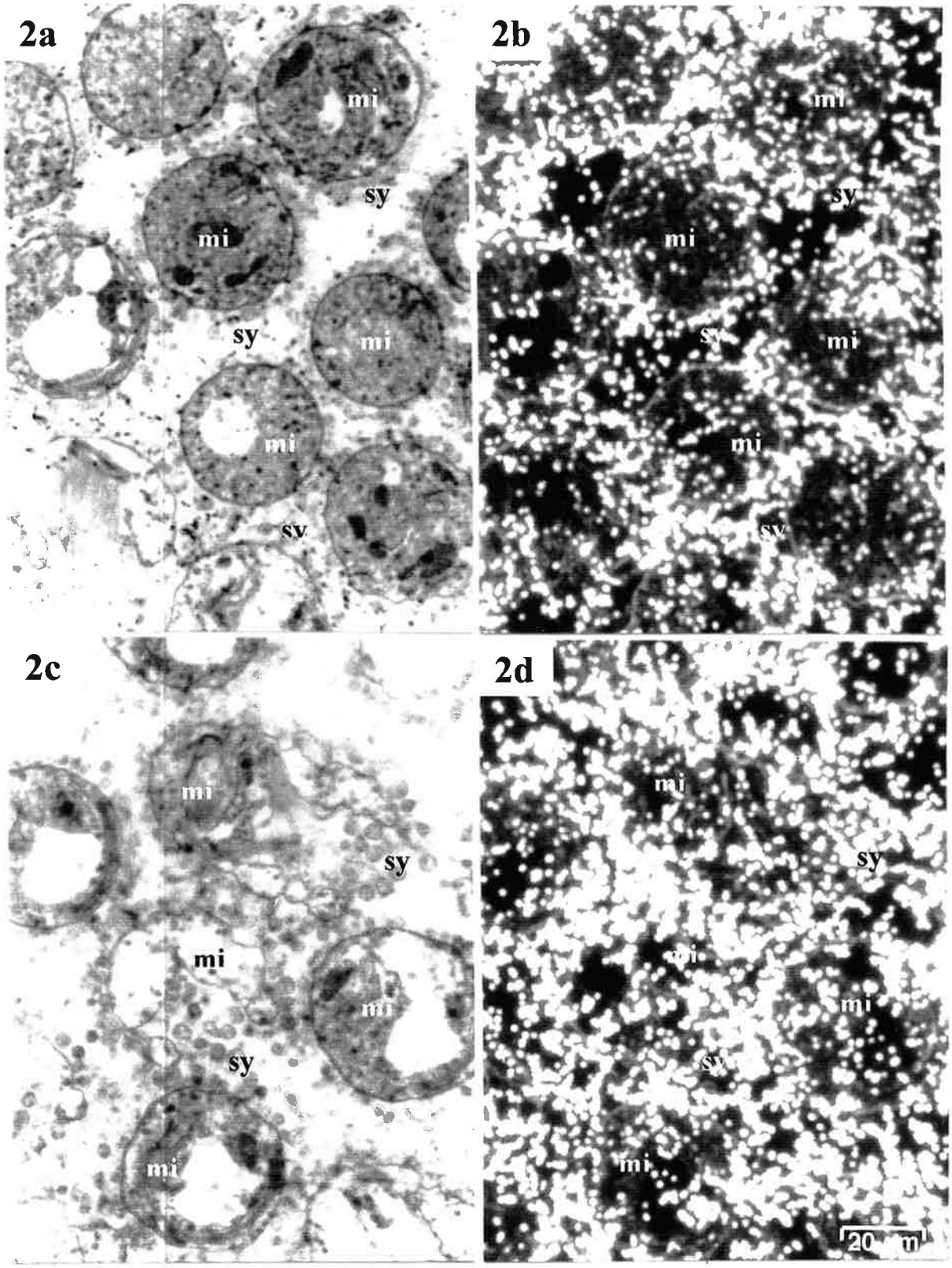

Fig. 2. *Zoothamnium niveum*. Light micrographs of sections through the colony, bright-field illumination (2a, 2c) on the left, dark-field illumination (2b, 2d) on the right side, showing microzooids of the host (mi) and ectosymbionts (sy). (2a, 2b) 15 min short pulse incubation in thiosulfatic seawater shows a dense label primarily over the ectosymbionts. (2c, 2d) 3 h hour long pulse incubation in thiosulfatic seawater shows a dense label over both partners, ectosymbionts and host.

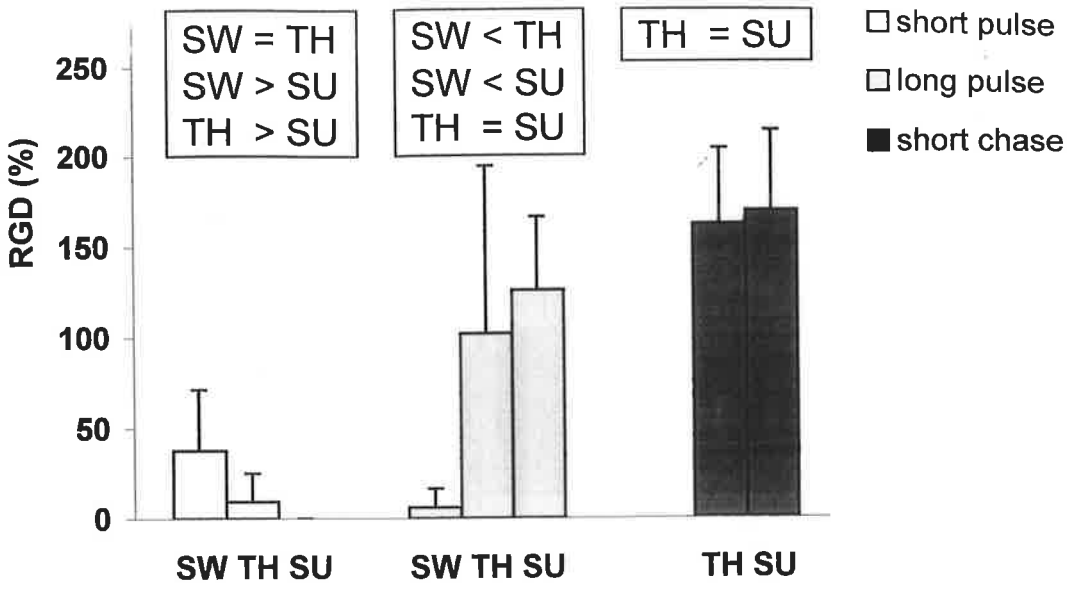

Fig. 3. *Zoothamnium niveum* specimens. Relative Grain Density (RGD) (mean ± standard deviation) over the host tissue after short pulse, long pulse and short chase under normoxic seawater (SW), thiosulfatic seawater (TH) and sulfidic seawater (SU) conditions. No bar means, that the label over the host tissue showed no significant difference to the background; = refers to no significant difference; > or < refers to significantly larger or smaller (post hoc Tamhane test, confidence level 99%).

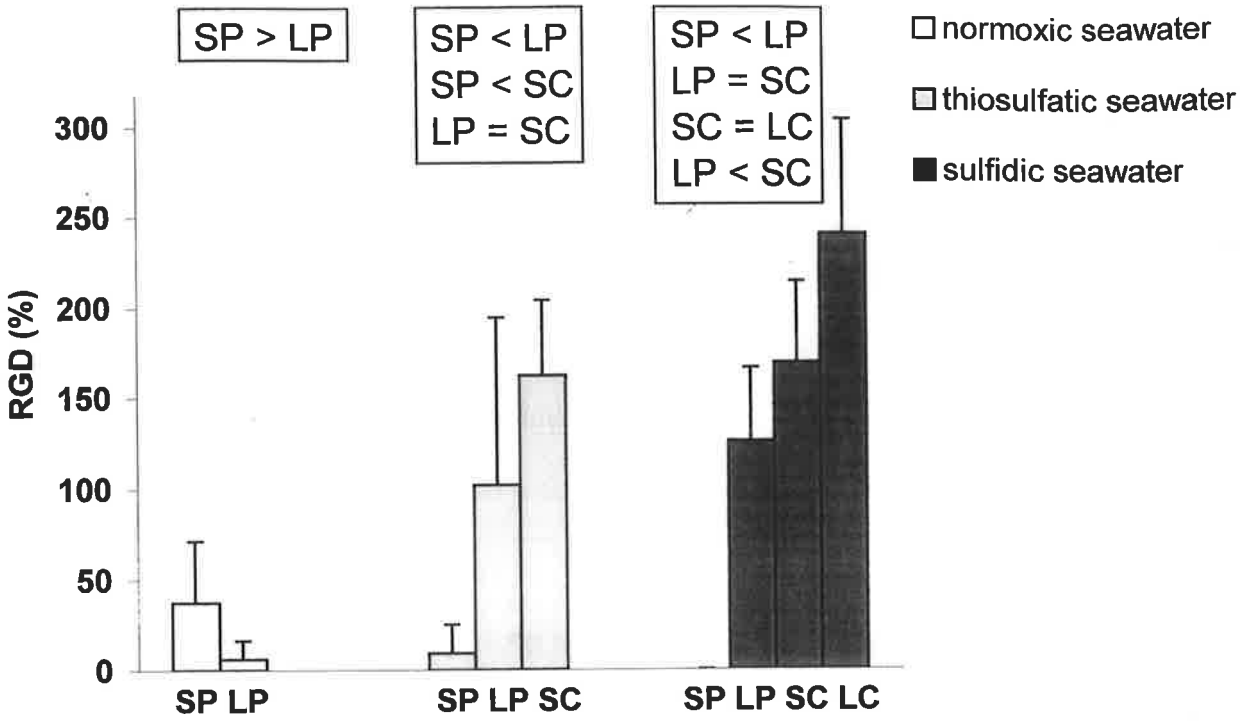

Fig. 4. *Zoothamnium niveum* specimens. Relative Grain Density (RGD) (mean ± standard deviation) over the host tissue after short pulse (SP), long pulse (LP), short chase (SC), and the long chase (LC) under normoxic seawater, thiosulfatic seawater and sulfidic seawater conditions. No bar means, that the label over the host tissue showed no significant difference to the background; = refers to no significant difference; > or < refers to significantly larger or smaller (post hoc Tamhane test, confidence level 99%).

LEGEND

of table 2

Table 2. *Zoothamnium niveum* specimens incubated in filtered seawater under different chemical conditions, normoxic, thiosulfatic ,and sulfidic seawater. Actual Grain Density (AGD) (mean ± standard deviation) is the number of silver grains counted over 10 x 20 µm of different tissues in 1 µm semithin sections with an exposure period of 9 days to autoradiography emulsion. The Relative Grain Density (RGD) (mean ± standard deviation) is set in proportion to the counts over the ectosymbionts (including the bacteria attached to microzooids, swarmers and stalk , weighted 33,3% each). Comparison between experiments with different time frames (short pulse, long pulse, short chase, long chase) were tested with the post hoc Tamhane test; cocci refers to the coccoid epibionts on the microzooids; ma-rods refers to the rod-shaped epibionts on the macrozooids; st-rods refers to the rod-shaped epibionts on the stalk; n (number) refers to the number of specimens for each treatment in which symbionts were autotrophically active; subs (subsamples) refer to the number of counted areas for each tissue; n/a refers to not applicable due to the death during incubation; =, not significantly different; > or < refer to significantly larger or smaller; -, not tested (confidence level 99%).

		short pulse (15 min)				long pulse (3 h)				short chase (3 h pulse + 12 h chase)				long chase (3h pulse + 24 h chase)			
		RGD	AGD	subs		RGD	AGD	subs		RGD	AGD	subs		RGD	AGD	subs	
ormoxic	**symbiotic**																
eawater	**tissue**	**100,00 ± 56,27**	**3.21 ± 2.18**	**48**	**-**	**100,00 ± 12,63**	**6.41 ± 0.79**	**45**									
	cocci	164,52 ± 45,84	5.68 ± 0.02	26	=	110,76 ± 29,60	7.02 ± 1.92	17									
P: n=3	ma-rods	61,08 ± 21,56	1.58 ± 0.35	13	=	103,14 ± 24,60	6.68 ± 2.13	11									
P: n=3	st-rods	74,40 ± 39,33	2.36 ± 1.79	9	=	86,10 ± 5,10	5.52 ± 1.02	17			n/a				n/a		
	host tissue	**37,67 ± 33,82**	**1.33 ± 1.21**	**48**	**>**	**6,06 ± 10,50**	**0.41 ± 0.71**	**45**									
	microzooids	47,59 ± 13,14	1.63 ± 0.88	26	=	18,18 ± 9,18	1.22 ± 0.72	17									
	macrozooids	0,00 ± 0,00	0.00 ± 0.00	13	=	0,00 ± 0,00	0.00 ± 0.00	11									
	stalk	65,42 ± 66,23	2.36 ± 2.77	9	>	0,00 ± 0,00	0.00 ± 0.00	17									
hiosulfatic	**symbiotic**																
eawater	**tissue**	**100,00 ± 9,29**	**4.44 ± 0.48**	**51**	**-**	**100,00 ± 17,66**	**2.16 ± 0.42**	**77**	**-**	**100,00 ± 17,63**	**2.85 ± 0.82**	**69**	**-**				
	cocci	90,72 ± 14,69	3.95 ± 0.39	23	=	91,14 ± 31,07	1.98 ± 0.96	36	=	119,19 ± 44,94	3.80 ± 3.53	28	=				
P: n=3	ma-rods	109,31 ± 17,96	4.92 ± 1.61	13	=	88,53 ± 29,51	1.86 ± 0.76	28	=	96,26 ± 45,44	2.47 ± 0.77	27	=				
P: n=4	st-rods	99,98 ± 5,23	4.43 ± 0.81	15	=	120,34 ± 28,59	2.65 ± 1.49	13	=	84,54 ± 44,38	2.30 ± 1.11	14	=		n/a		
C: n=4	**host tissue**	**9,24 ± 16,00**	**0.41 ± 0.71**	**51**	**<**	**102,28 ± 92,15**	**1.87 ± 1.66**	**77**	**=**	**162,17 ± 41,90**	**4.13 ± 1.30**	**69**	**>**				
	microzooids	27,71 ± 13,07	1.23 ± 0.67	23	<	128,01 ± 49,34	2.42 ± 0.59	36	=	143,48 ± 47,70	4.27 ± 3.46	28	>				
	macrozooids	0,00 ± 0,00	0.00 ± 0.00	13	<	178,83 ± 86,55	3.18 ± 1.31	28	=	210,16 ± 22,29	5.36 ± 2.04	27	>				
	stalk	0,00 ± 0,00	0.00 ± 0.00	15	=	0,00 ± 70,50	0.00 ± 0.00	13	<	132,87 ± 66,21	2.78 ± 2.73	14	>				
sulfidic	**symbiotic**																
eawater	**tissue**	**100,00 ± 26,67**	**2.99 ± 0.71**	**38**	**-**	**100,00 ± 21,73**	**1.66 ± 0.34**	**77**	**-**	**100,00 ± 14,71**	**1.71 ± 0.27**	**70**	**-**	**100,00 ± 24,26**	**1.34 ± 0.32**	**129**	
	cocci	126,70 ± 30,63	3.63 ± 0.63	20	=	112,93 ± 31,02	1.87 ± 0.69	33	=	99,94 ± 10,24	1.67 ± 0.53	30	=	83,91 ± 37,38	1.08 ± 0.39	48	
P: n=3	ma-rods	73,35 ± 5,63	2.22 ± 0.77	9	=	75,00 ± 32,29	1.27 ± 0.79	19	=	85,32 ± 33,95	1.47 ± 0.97	12	=	88,19 ± 39,83	1.25 ± 0.71	42	
P: n=4	st-rods	99,94 ± 26,37	3.11 ± 1.51	9	=	112,33 ± 13,48	1.85 ± 0.66	25	=	114,74 ± 33,04	2.00 ± 1.05	28	=	127,90 ± 36,81	1.69 ± 0.51	39	
C: n=3	**host tissue**	**0,00 ± 0,00**	**0.00 ± 0.00**	**38**	**<**	**125,92 ± 40,24**	**1.90 ± 0.65**	**77**	**=**	**169,13 ± 45,05**	**2.55 ± 0.66**	**70**	**=**	**240,27 ± 63,82**	**3.15 ± 0.82**	**129**	
C: n=5	microzooids	0,00 ± 0,00	0.00 ± 0.00	20	<	172,39 ± 57,07	2.65 ± 0.44	33	=	220,99 ± 112,00	3.28 ± 0.13	30	=	297,49 ± 107,02	3.86 ± 1.13	48	
	macrozooids	0,00 ± 0,00	0.00 ± 0.00	9	<	103,23 ± 51,54	1.58 ± 0.67	19	=	139,69 ± 27,37	2.33 ± 0.93	12	=	251,89 ± 56,57	3.34 ± 0.95	42	
	stalk	0,00 ± 0,00	0.00 ± 0.00	9	<	102,14 ± 56,15	1.47 ± 0.31	25	=	146,72 ± 109,09	2.03 ± 0.60	28	=	171,44 ± 51,61	2.25 ± 0.66	39	

DISCUSSION

Survival and chemoautotrophy under different chemical conditions. Short time incubation experiments for up to 27 hours under different chemical conditions give insight into the physiological behavior of the symbionts and the host. This valuable information can be used in more directed attempts for cultivation of this symbiosis. So far, thiotrophic symbiosis research is extremely limited due to the fact that none of the symbioses have been cultured under axenic conditions successfully. To the contrary of other symbioses in which the habitat such as the deep sea hydrothermal vents is difficult to mimic due to the necessity of using high pressure flow through aquaria (for example *Riftia pachyptila*, see Goffredi et al. 1997) and the comparably slow growth rates and life cycles of most invertebrate hosts compared to protists, *Zoothamnium niveum* from a shallow water habitat should be an ideal candidate for cultivation. Indeed, during the past years we were able to maintain and culture this symbiosis under "natural" conditions for a few successive generations (Bright, Ott pers. obs.). *Z. niveum* not only exhibits a life cycle with short generation time and fast reproduction, it also reacts to changes in physico-chemical conditions quickly in either surviving and reproducing or dying.

While *Zoothamnium niveum* symbiosis died under normoxic conditions between 3 hours and 27 hours incubation time, it survived that period of time when 1 mM thiosulfate was added. The symbiosis survived a longer time period by incubating in 100 µM ∑sulfide for the first 3 hours and subsequent incubation in 1 mM thiosulfate for 24 hours. The viability of the host was evaluated by observing the contraction/expansion behavior of the colony in combination with the presence or loss of individual host cells and the ciliary activity of the microzooids. The symbionts were evaluated by their color. In many other thiotrophic free-living and symbiotic bacteria the white color is due to elemental sulfur inclusions (Prange et al. 1999, Arndt et al. 2001). When an external reduced sulfur species is not available, this

internal storage compound will be used in chemosynthesis and the bacteria turn pale gradually. Thus, the color of the symbionts does not indicate the viability but the chemoautotrophic potential (Fig. 5).

This autoradiography study using ^{14}C bicarbonate in pulse/chase experiments clearly demonstrated that the *Zoothamnium niveum* symbiosis was autotrophically active (Fig. 2). A radioactive inorganic carbon source was added to the incubation media and later showed up as high molecular weight organic carbon over symbiotic and host tissues (see Rogers 1979). This matches with earlier investigations in which one of the key enzymes in the Calvin-Benson cycle , ribulose-1,5-bisphosphate carboxylase/oxygenase was detected (Felbeck pers. comm.). During the 3 hours incubations in SW, the symbionts remained their white color. The incorporation of organic carbon is hypothesized to be due to the use of elemental sulfur storage. The results are in accordance with Cartesian diver experiments that demonstrated the use and rapid consumption of this internal storage by fast decrease in oxygen consumption rates. It was shown to drop to less then half of the initial rate after 3 hours in SW indicating that the internal elemental sulfur products provide energy for carbon fixation for about 4 hours (Ott et al. 1998). Additionally, earlier cultivation experiments confirm these findings and showed that within a maximum of 12 hours the symbionts turned pale and the host degenerated (Bright, Ott pers. obs.).

During all incubations with added reduced sulfur species, the symbionts remained white, indicating that, in all experiments lasting more than 3 hours, the symbionts did not access their internal sulfur storage but used the external thiosulfate or sulfide pool. Thus, incorporation of organic carbon that could be followed directly for the 3 hours pulse period was evident. While the host survived and the symbionts stayed white for 27 hours in SU, death of the host occurred in TH between 15 and 27 hours. We encountered this phenomenon numerously earlier. Usually, death happens in thiosulfatic seawater within a maximum of 3

days. However, the symbionts always remained white, even after the host died, leaving behind a cloud of white substance on the bottom of the vials (Bright, Ott pers. obs.).

Symbiotic behavior. Many thiotrophic symbioses are strict two-partner associations with a single bacterial phylotype. The findings of more than one morphotype with transitional stages and/or fluorescence in situ hybridizations using symbiont specific oligonucleotid probes suggest that in some symbiotic phylotypes more than one morphotype is simultaneously present. This points to a complex bacterial life cycle according to Dworkin (see Bright 2002). *Riftia pachyptila* (Vestimentifera) the giant tubeworm from the hydrothermal vents harbors a monospecific bacterial population, consisting of 3 endosymbiotic morphotypes, rods, small cocci and large cocci (see Gardiner and Jones 1993). The morphologically different population of filamentous bacterial epibionts on the atlantic vent shrimp *Rimicaris spp.* was analysed to be a single phylotype. *Zoothamnium niveum* symbionts exhibit smaller rods and larger cocci with transitional stages in between (Bauer-Nebelsick et al. 1996a, b). This study revealed that the single cell incorporation rate of the two morphotypes is different despite the fact that the incorporation rates per area over both symbionts are similar. The average length of the cocci is 1.9 ± 0.3 µm (diameter 1.0 ± 0.4 µm) and that of the rods is 1.4 ± 0.2 µm (diameter 0.4 ± 0.0 µm) (see Bauer-Nebelsick et al. 1996b). Even if we consider that in the sections used bacteria were cut by pure chance thus leading to a mixture of sections with all possible directions and a maximum of length and diameter as given above, it can be safely assumed that a single coccus incorporates significantly more than a single rod.

Also the endosymbiotic morphotypes of *Riftia pachyptila* do not incorporate organic carbon to the same degree. Bright et al. (2000) suggested that these differences in organic carbon incorporation reflect differences in life cycle stages and metabolism in which a small proportion of the symbiotic population is rod-shaped and able to divide and the remaining coccoid population is much larger and does not divide any more. In the *Zoothamnium niveum*

symbionts, although rods and cocci are present as well, both populations are dividing in the manner of a simple life cycle each (see Bright 2002).

We suggest that the reasons for the metabolic difference between cocci and rods in the *Zoothamnium niveum* symbionts is due to the behavior and the morphology of the host that creates different microenvironments with chemical conditions that support higher fixation rates, higher growth rates and larger cells for the orally located cocci than for the rods on the remaining parts of the colony. The host ciliate supplies the symbionts with oxygen and sulfide by contractions/expansions of the entire colony (Ott et al. 1998) as well as by the feeding current of the oral ciliature of the microzooids (Vopel et al. 2001). The intervals between two successive contractions range from 5 to 30 seconds. The contractions are very fast and take less than four hundreds of a second and are followed by a period of less than a second in which the colony remains contracted. The following expansions are very slow and take 1 to 3 seconds in which the sulfidic water is carried along upwards with the expanding colony into oxic layers. After the colony reaches the upright position, the microzooids immediately start to vortex, producing a water current that mixes the sulfidic and oxic water around the oral ciliature very fast. The much larger macrozooids, although able to vortex too, have a comparably small oral ciliature and were found to show less ciliary activity (Bright pers. obs.). Thus, the cocci from the oral area of the microzooids are supplied with oxygen faster than the rods on the remaining parts of the colony. This leads to microenvironments with temporal differences in oxygen and sulfide supply. We hypothesize that the temporal ratio between oxygen and sulfide is favorable in the oral area and leads to a higher fixation rate of the symbionts there, which then can grow faster and become larger. The lack of fast oxygen supply for example around stalk and branches, on the other hand, is hypothesized to support only lower fixation rates, thus the symbionts there grow slower and remain smaller.

Nourishment of host. The symbionts are the site of carbon fixation, demonstrated by high levels of incorporation after short pulse incubations, they incorporate a portion of the

organic carbon they fix and may use it for respiration, growth, or division. Part of the organic carbon is released and taken up by the host colony shortly after fixation. Additionally, the host's microzooids actively feed on their symbionts by phagocytosis. A portion of the digested organic carbon the microzooids use themselves, another portion is translocated to the non-feeding host cells such as the macrozooids and the supportive structures of the colony such as the stalk and the branches (Fig. 6).

The detection of labeled organic carbon in the heterotrophic host tissue is plausible evidence that the ciliates are nourished by their symbionts. The transfer of fixed carbon from the symbionts to the host is hypothesized to occur via release of low molecular weight compounds and via vortexing of oral cilia, ingestion, and digestion of whole symbiont cells. The label over host tissue only after 15 min incubation time provides information about the pathway the host acquires parts of the symbionts' carbon pool. The digestive cycle of some ciliates such as the bacteriovor and algivor *Paramecium* (Nassophora) and *Tetrahymena* (Oligohymenophora) are well known. The entire process from food ingestion to the time when the digestive can be used to gain ATP or to incorporate it in structural tissue takes 30 to 50 min in *Paramecium* (Allen and Fork 1980, 1983 a, b, 1993) while it takes up to 3 hours in *Tetrahymena* (Vosskühler and Tiedke 1993). These studies support our hypothesis that label over host tissue after 15 min is due to fast uptake and incorporation of products released quickly by the symbionts after fixation. For the gutless bivalve *Solemya reidi*, the translocation of fixed and nearly simultaneously released low molecular weight compounds by chemoautotrophic endosymbionts is suggested to be the major mode of carbon transfer in this association. However, digestion of symbionts was not excluded either (Fisher and Childress 1986). The host in the *Riftia pachyptila* symbiosis derives nutrition from compounds released by the endosymbionts as well as through digestion of their symbiotic bacteria (Bright et al. 2000). The same carbon pathway was suggested for the littoral bivalve *Loripes lucinalis* and their chemoautotrophic endosymbionts (Herry et al. 1989). Through this

mode of carbon transfer more than 45% of the carbon fixed by the symbionts of *S. reidi* and is released and taken up by the host clam (Fisher and Childress 1986). Taking into account that all light molecular weight products released from endosymbionts could theoretically be utilized by the surrounding host, a release of products by ectosymbionts into the surrounding water leads to a fast dilution. Thus, the possible amount of uptake of products released by the epibionts can be assumed to be lower in ecto- than in endosymbioses.

Ingestion and phagocytosis of the bacteria is an important nutritional process in the *Zoothamnium niveum* symbiosis. The RGDs over host tissue doubles from the long pulse to the long chase period in SU incubation media. During the 24 hour chase period, the symbionts had no radioactive inorganic carbon source and therefore, were not able to provide a radioactive release for the host. Thus, only digestion of symbiotic cells followed by incorporation into host tissue can explain the increase of label over host tissue. Based on the assumption that the macrozooids are not able of phagocytosis due to a reduced cytopharynx (Bauer-Nebelsick et al. 1996 b) and that the stalk and the branches lack digestive structures entirely, the label over the macrozooids and the stalks can only be explained by uptake and incorporation of bacterial release products or by carbon transfer from the feeding microzooids. Looking again at the label over host tissue after long pulse and long chase periods, a significant increase over macrozooids and stalk was found. Again symbiont release cannot explain that increase due to the lack of a radioactive source for carbon fixation during the long chase period. Therefore, we suggest that transfer or organic carbon, digested by the feeding microzooids, occurs via cytoplasmic connections that exist between all host cells and supportive structures (Fig. 6).

Conclusion. Through short time autoradiography experiments using *Zoothamnium niveum* and its ectosymbiotic bacteria, we could show that the symbionts are autotrophic organisms. The symbionts are the site of chemoautotrophic carbon fixation using the oxidation of the internal elemental sulfur storage or an external provided pool of sulfide or

thiosulfate in order to gain energy for carbon fixation. We could clearly demonstrate that the host is nourished by two modes of nutrition, uptake of low molecular weight products and incorporation and by digestion of ingested symbionts. Under natural conditions, it can be safely assumed that by actively feeding, the microzooids not only ingest their own symbionts, but also a variety of free-living bacteria from the water column. The partial ratios of free-living bacteria and symbionts and the partial ratio of release and digestion on the entire carbon uptake need further investigation.

Fig. 5. *Zoothamnium niveum*. An aggregation of colonies on a mangrove peat wall in the tidal channel of the Caribbean island Twin Cays (Belize Barrier Reef). The black arrow points to white colonies, the white arrows point to pale colonies.

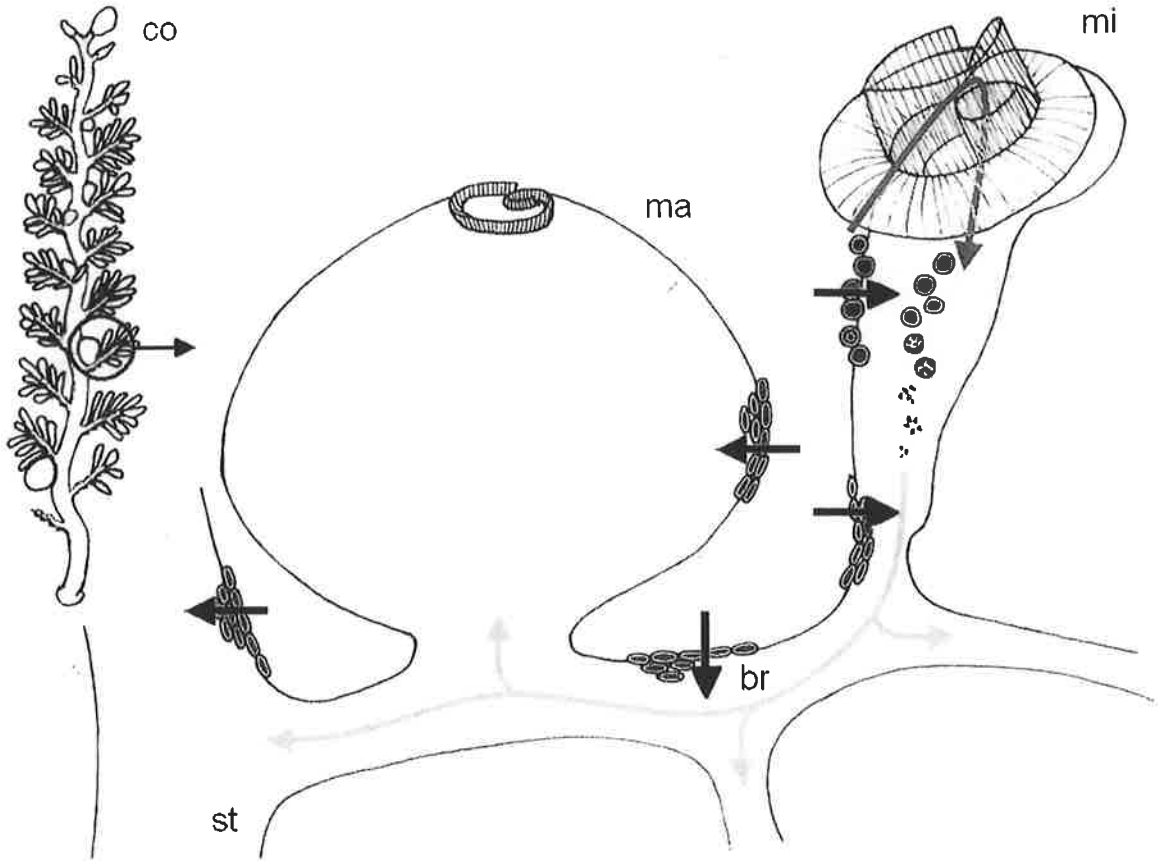

Fig. 6. Proposed model of organic carbon pathways in the *Zoothamnium niveum* symbiosis. Schematic drawing of the entire colony (co) on the upper left side and a close-up of a macrozooid (ma) and a microzooid (mi) connected by a branch (br) and a part of the stalk (st). The transfer of fixed carbon from the bacteria to the host occurs via release of low molecular weight compounds (black arrows) by the rod-shaped bacteria on macrozooids, branch and stalk as well as by the coccoid bacteria attached to the microzooid, or by ingestion and digestion (grey arrow) of bacterial cells by the microzooid. The organic carbon is transferred from the microzooid to branch, stalk and macrozooid (light grey arrows).

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ZUSAMMENFASSUNG

Der große, sessile, schnellwüchsige und koloniale Ciliat *Zoothamnium niveum* (Ciliophora, Oligohymenophora) bewohnt die vertikalen Torfwände in den Gezeitenkanälen der Mangroveninseln im Barriere Riff von Belize. Bestimmte Bakterien, die den Ciliaten fast vollständig umhüllen, sind für den Wirt dieser Symbiose obligat erforderlich. Erste Anzeichen einer Nahrungsbeziehung stammen von Beobachtungen an lebenden Individuen, die Bakterien ingestierten. Diese Bakterien waren den eigenen Symbionten ähnlich. Außerdem zeigen Ultradünnschnitte des Wirts diese morphologisch ähnlichen Bakterien in Nahrungsvakuolen im Stadium der Verdauung. Die Aufgabe dieser Studie war, die Kohlenstofffixierung und Inkorporation der autotrophen Symbionten unter unterschiedlichen chemischen Bedingungen und Zeitabfolgen mittels ^{14}C Bikarbonat Autoradiographie zu untersuchen. Außerdem sollten die Umstände eines möglichen Transfers von fixiertem Kohlenstoff von den Symbionten zum Wirt sowie die Verteilung des organischen Kohlenstoffes in den Wirtszellen festgestellt werden.

Unseren Ergebnissen nach sind die Symbionten bei Inkubationen in sauerstoffreichem, thiosulfatischem und sulfidischem Seewasser fähig, anorganischen Kohlenstoff zu fixieren und organischen Kohlenstoff zu inkorporieren. Ebenfalls festgestellt wurden Inkubationen in Seewasser ohne Zusatz von Schwefelverbindungen. Dies ist wahrscheinlich durch einen Speicher von elementarem Schwefel in den Symbionten möglich. Experimente mit „Kartesischen Tauchern", die die Oxidation von elementarem Schwefel innerhalb weniger Stunden zeigen, bestätigen diese Schlussfolgerung.

Die Bakterienpopulation besteht aus zwei verschiedenen Morphotypen, den Kokken und den Stäbchen, die unterschiedliche Mengen an Kohlenstoff pro Zelle inkorporieren. Dies ist durch eine unterschiedliche Sulfid- und Sauerstoffversorgung der Bakterientypen zu erklären, die durch das Verhalten und die Morphologie des Wirts zustande kommt.

Der Transfer des Kohlenstoffs von den Symbionten zum Wirt erfolgt zum einen über die Verdauung der Symbionten, zum anderen über die direkte Aufnahme von niedermolekularen Stoffen, die von den Bakterien abgegeben werden. Diese schnell nutzbaren, bakteriellen Abgabestoffe waren bereits nach 15 Minuten radioaktiver Inkubation zu sehen. Der langsamere Verdauungsprozess zeigte sich in einem Anstieg des radioaktiven Signals im Wirt von der 3 Stunden zur 24 Stunden Inkubation. Eine Translokation der verdauten Nährstoffe von den fressenden Mikrozooiden zu den nicht fressenden anderen Teilen der Kolonie, wie Makrozooiden, Stiel und Ästen, erfolgte rasch. Das radioaktive Signal konnte zur selben Zeit detektiert werden. Wahrscheinlich verläuft dieser Transfer über zytoplasmatische Verbindungen.

Unsere Experimente beweisen deutlich eine enge Nahrungsbeziehung zwischen den thiotrophen Ektosymbionten und *Zoothamnium niveum.* Der Ciliat als Wirt kann sowohl den von Bakterien abgegebenen, niedermolekularen, organischen Kohlenstoff aufnehmen, als auch seine eigenen Symbionten einstrudeln und verdauen. Zusätzlich konsumiert der Wirt frei lebende Bakterien aus dem Umgebungswasser. Um den genauen Anteil der Symbionten in der Nahrung des Wirts zu bestimmen, bedarf es weiterer Untersuchungen.

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