Chemosynthetische Endosymbiosen an rezenten und fossilen Cold Seep-Standorten“

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1. General Introduction

Cold seeps are seafloor sites characterized by releasing reduced sulfur and methane from the sediment; these sites occur worldwide at active and passive continental margins (Fig. 1) and in some cases also in freshwater lakes. They are found in the deep sea as well as in shallow-water (from depths of less than 15 m to 7400 m) and are called 'cold' because other than in hot vents, there is no exchange with hydrothermal systems (Paull et al., 1984; Sibuet & Olu, 1998; Fujikura et al., 1999; Kojima, 2002; Levin, 2005).

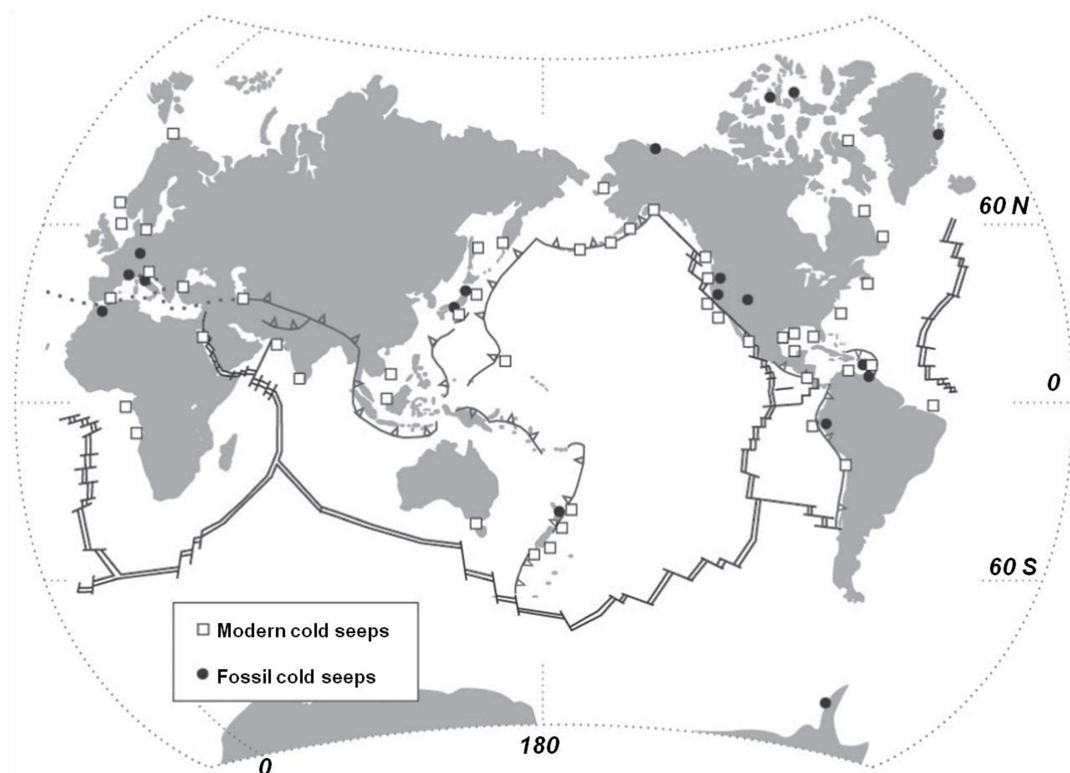

Fig. 1. Distribution of modern and fossil cold seeps. (From Levin, 2005; modified from Campbell et al., 2002)

Seafloor sediments consists of carbonate sands, quartz sand, clays or muds and also terrestrial turbidites (Levin, 2005). At seep sites, hard concretions (carbonate precipitations arise within the soft sediment and build different structures like mounds and platforms (Barbieri & Cavalazzi 2004). Microbes populate cold seeps using reduced fluids as energy sources and play a major role in the buildup of these carbonate formations. Furthermore, the bacterial

biomass fuels a whole seep ecosystem of marine invertebrates. Most of these invertebrates, inter alia mussels, clams and tubeworms, evolved obligate symbioses with sulfur- and methane-oxidizing bacteria.

It was documented for seep sites below 400 m water depth that *c.* 30 % of found species were symbiont bearing (Sibuet & Olu, 1998). Relatives of symbiont bearing (‘chemosynthetic’) seep species could also be found at hydrothermal vents, anoxic basins, on whale- and wood-falls, and also in costal reducing sediments (Sibuet & Olu, 1998; Tunnicliffe et al, 1998; Smith & Baco, 2003).

The occurrence of chemosynthetic metazoan species or of a whole chemosynthetic ecosystem are indicators for reducing conditions in the respective environment. The reconstruction of environmental and climatic conditions and changings during Earth history is a wide field of recent investigations (Sheldon & Tabor, 2009; Eiler, 2011; Wanamaker et al., 2011; Yang et al., 2011). The occurrence of fossil chemosynthetic animals at fossil marine settings helps to deduce redox conditions in ancient sediments. Also the influence of environmental conditions on the evolution of symbiont-bearing invertebrates is still a big question. Does the symbiosis evolve before the animals colonized a seep region or do seep areas trigger the evolution of bacteria-invertebrate interaction?

To solve these questions we need signatures which help us to determine possible symbiosis in fossil species. By this way, it might be even possible to discover changing feeding behavior of different species in a phylogenetic group. Until now many different fossil vent and seep settings with their characteristic fauna are explored (Fig. 1; Haymon et al., 1984; Paull, 1984; Gaillard & Rolin, 1986; Niitsuma et al., 1989; Kuznetsov et al., 1993; Little et al., 1997; Majima et al., 2005; Campell, 2006; Kiel, 2006; Kiel & Peckmann, 2008).

Finding symbiotic bacteria in living seep and vent species is not that difficult. A plethora of established techniques are available to detect and identify symbionts in situ, such as 16S rDNA analysis, fluorescence *in situ* hybridization (FISH) imaging or electron microscopy. Solving the question if an extinct animal relied on symbionts during its lifetime is a difficult challenge. Morphological features of fossil species and analysis of facies from surrounding area were compared to

their recent relatives, assuming that same species in same environment live likewise. In the past few years new tools were developed and molecular fossils or geo- and biochemical markers were used to discover ancient seeps and vent and the related fauna (see Chapter 2).

One prominent group at vent and seep sites are mollusks, especially bivalves. Most of those bivalves harbor chemoautotrophic bacteria which oxidize sulfide, they are 'thiotrophic'. Other bivalves are known to have a relationship to methane oxidizing bacteria and some bivalves have a symbiotic relationship to both methane and sulfur-oxidizers.

Chapter 2 of this thesis presents a review about recent and ancient symbiont bearing mollusks (with focus on bivalves) and about common methods of 'molecular paleontology' which are used to determine putative ancient symbiosis between bivalves and bacteria.

As pointed out in **Chapter 2**, the occurrence of thiotrophic bivalves is not restricted only to vent and seep sites. We find some of them also in reducing sediments (non-seep sites) of shallow waters at the oxic/anoxic interface. The concentration of sulfide at non-seep sites is much lower (µM range) than in seep sediments (up to mM range; Bagarinao, 1992; Somero et al., 1989). The population of symbiotic bivalves here is not as dense as known from seep and vent sites but symbiont bearing bivalves from shallow water sediments are more easy to sample. In most cases additional non-symbiotic bivalves occur at non-seeps sites as well. Thus, these sites are excellent settings for comparative analysis of different symbiotic and non-symbiotic bivalve species.

Chapter 3 focuses on the comparison of biochemical and isotopic signatures between a symbiotic and a non-symbiotic bivalve from a non-seep site. This investigation was also done in view of the origin of those signatures and their preservation over geological time.

If we want to declare an ancient fossil bivalve as symbiotic, we have to keep in mind that in shallow water not only thiotrophic and non-symbiotic bivalves occur. As a third variant, some bivalves may harbor symbiotic dinoflagellates using photosynthesis instead of sulfide to gain reducing power for carbon fixation. This may be also relevant for important extinct bivalve groups.

As reviewed in **Chapter 2** some authors speculate about an ancient phototrophic lifestyle of some bivalves inter alia members of the extinct group of Inoceramidae.

Thus, the aim of **Chapter 4** is to report the differentiation between biochemical signatures from bivalves which bear thiotrophic symbionts and those with a symbiotic relationship to phototropic algae. In addition, **Chapter 4** deals with the question of quality and long term conservation of the organic shell matrix, in particular with respect to the preservation of biochemical markers.

Chapter 5 is a general discussion and conclusion, also summarizing the most important results of Chapter 3 and 4.

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2. Following the traces of symbiont bearing mollusks during earth history

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2.1 Abstract

Ivan E. Wallin (1883-1969) was among the first scientists who noticed the evolutionary impact of symbiotic events. He proposed that endosymbiosis was the principal source for speciation (Wallin 1927). Mitochondria and chloroplasts as symbiotic descendants of bacteria in a eukaryotic cell are the well-known and most important endosymbiotic key players, enabling and shaping the evolution of eukaryotes. In addition, a multitude of other symbioses between prokaryotes and Eukarya have been described so far. As an example, symbiosis between mollusks and sulfur- or methane-oxidising bacteria, is a widespread lifestyle in marine habitats (and perhaps yet undetected for other environments). These symbiotic associations occur worldwide at oxic–anoxic interfaces such as at the boundary layer of reducing sediments, in cold seeps, in hydrothermal vents or in mangrove peat. The symbiosis between marine mollusks and chemosynthetic bacteria increase the metabolic capabilities and therefore the possibilities to occupy ecological niches of both host and symbiotic prokaryote. Nowadays, due to molecular analysis and in situ hybridisation techniques, detection of symbioses in recent living organisms is not that difficult. But finding a path back to the point in Earth's history were symbiotic events took place is a tricky challenge.

Not long ago only analyses of morphological features of shells and facies criteria were available for assessment of the lifestyle and the diet of extinct bivalves. Close phylogenetic relationships to recent symbiont bearing genera in a similar habitat make it likely that the extinct genera exhibited a similar lifestyle, but these indirect criteria are not sufficient to uncover ancient symbiosis in mollusks.

In this review several approaches of "molecular palaeontology" are discussed, which allow for a direct determination of a symbiotic or non-symbiotic lifestyle in recent and fossil mollusks.

2.2 Introduction

"It is concluded that the evolutionary potential of symbiosis is great and that symbiosis serves as a supplementary speciation mechanism capable of producing directed evolutionary changes" (Taylor, 1979). This conclusion may be particularly true for bivalves: Bacteria and marine mollusks, often form mutualistic partnerships which markedly influence the physiology, ecology and evolution of both.

Autotrophic bacteria assimilate inorganic carbon as primary carbon source. The bacterium is chemoautotrophic when reducing power and energy needed for assimilation of carbon dioxide derives from reduced inorganic compounds. The other important energy source is sunlight for photoautotrophic organisms. Chemoautotrophic or methanotrophic bacteria are found in a wide range of reducing habitats providing these coveted energy sources, in particular H_2, H_2S or CH_4. Most prominent sites are cold seeps and hydrothermal vents, but dysoxic conditions are frequent in marine environments, such as in seagrass beds, mangrove sediments or wood and whale falls. These habitats of free living autotrophic or methanotrophic bacteria are usually also inhabited by mollusks hosting symbiotic chemoautotrophs or methanotrophs (Lonsdale, 1977; Corliss et al., 1979; Jannasch & Wirsen, 1979; Van Dover, 2000; Treude et al., 2009; Kiel & Tyler, 2010). Though also bacteria of other metabolic types are symbionts of marine invertebrates, most of them are sulfur-oxidisers or methanotrophs belonging to the Gammaproteobacteria. According to

phylogenetic analysis these symbioses have been established multiple times in earth history and evolved independently (Dubilier et al., 2008).

Several marine mollusks, especially some species of Cephalopoda, Gastropoda and Bivalvia, are known to cultivate symbiotic microbes. The basic feature of this relationship is that the symbionts need reduced substrates and electron acceptors for their metabolism, which do not co-occur in the same microenvironments (Zhang & Millero, 1993). The mollusks are able to bridge the oxic–anoxic boundaries using behavioral, morphological or metabolic adaptations and supply substrates (e.g., reduced sulfur compounds) and electron acceptors (oxygen in most cases) to the microbes. In turn, most if not all organic carbon and also nitrogen compounds are provided by the symbiont (Cavanaugh et al., 2006; Dubilier et al., 2008).

Photosymbiosis is most successful in oligotrophic water under nutrient-limited conditions (Hallock & Schlager, 1986; Hallock, 1987; Schlager, 2003). Eukaryotic algae of the genus *Symbiodinium* (zooxanthellae) are the most prevalent symbionts of mollusks. The zooxanthellae satisfy a major part of the host's energy demand (Trench et al., 1981; Klumpp et al., 1992; Hawkins & Klumpp, 1995). In turn, zooxanthellae cover their nitrogen and phosphate demands mainly through their host’s excretion products.

Multiple studies provided insight into symbiont-host interaction, their metabolic features and how symbiotic partners are adapted to each other. Various approaches like 16S ribosomal DNA sequence analysis, fluorescence in situ hybridisation, transmission electron microscopy, stable isotope and fatty acid analysis were applied so far (Kharlamenko et al., 1995; McKenzie et al., 2000; Colaco et al., 2007). However, studies aiming at reconstruction of evolution of symbiotic mollusks were based on comparison of shell morphologies or were conducted in specific palaeoenvironments like vents and seeps (Fig. 1). In order to get a better understanding of the evolutionary steps and to give an estimate for the time point when a representative of a mollusk taxon starts its cooperation with microbes and shifts its diet to chemo- or phototrophic we need to detect a direct symbiotic fingerprint of the investigated fossil. These fingerprints or biosignatures must be stable in geological timescales.

Fig. 1. Late Miocene seep at Montepetra (Italy) with mass occurrence of lucinid clams (Heterodonta: Lucinoida).

2.3 Endosymbiotic Mollusks

2.3.1 Recent situation

Within the clades of recent mollusks, endosymbiosis with sulfur- or methane-oxidising (chemosynthetic) bacteria occur in seven bivalve families: Solemyidae, Nucinellidae, Montacutidae, Mytilidae, Thyasiridae, Lucinidae (being the most diverse familiy), Vesicomyidae (Taylor & Glover, 2009; 2010; Taviani, 2011; and references therein; Taylor et al., 2011; Oliver et al., 2013). In addition, the Teredinidae are known to harbour endosymbiotic cellulose-digesting symbionts (Distel et al., 2002).

The symbiosis seems to be obligate in all species of Lucinidae, Vesicomyidae and Solemyidae, while some species of Thyasiridae and Mytilidae are asymbiotic. Their life styles are highly diverse, ranging from epifaunal to deep infaunal (Taylor & Glover, 2010). Lucinids occur also in the deep sea at cold seeps (Callender & Powell, 1997), hydrothermal vents (Glover et al. 2004) and wood or whale falls (Dubilier et al., 2008). Though they appear to be rare on such sites, Kiel & Tyler (2010) stated that this might be a sampling artifact. Nevertheless, the more common mollusks of deep-sea habits are all three families of chemosynthetic gastropods, bathymodiolian mussels, vesicomyid clams and solemyids (Dubilier et al., 2008; Kiel & Tyler, 2010).

A symbiotic relationship to chemosynthetic bacteria is also known from three gastropod families (Provannidae, Lepetodrilinae and Peltospiridae) and from one family of the Aplacophora (Simorthiellidae, Dubilier et al., 2008). These organisms are all inhabitants of deep sea seep and vent sites.

Deep sea hydrothermal vents with their rich and constant supply of reduced inorganic compounds are perfect niches for a chemosynthetic lifestyle, which leads to mass development of chemosymbiotic mollusks in such environments. They are less abundant in the photic zones. The primary production in shallow water is driven by phototrophy and is usually dominated by heterotrophic communities; however in some cases chemosymbionts could also dominate in shallow water (Dando & Southward, 1986; Little et al., 2002; Tarasov et al., 2005). Though, the role of chemosynthetic mollusks in shallow water systems like coral reef sediments, seagrass meadows or mangrove sediments is not that unimportant. The evolutionary radiation of Lucinidae, for example, seems to be

linked to the emergence of seagrasses in the late Cretaceous (Van der Heide et al., 2012; and references therein). Lucinids are very important for the stability of seagrass systems, because they detoxify the surrounding sediment from sulfide and lead to oxygenation, with a not negligible effect on seagrass (Van der Heide et al., 2012). The highest diversity of recent Lucinidae was described for tropical reefal habitats (Glover & Taylor, 2007); also some solemyids (Krueger et al., 1996) and thyasirids (Dubilier et al., 2008) occur in this environment as well as some photosynthetic Cardiidea and Tridacnidea.

Some bivalve species within the Trapeziidae and Cardiidea (Fig. 2) maintain symbiotic associations with *Symbiodinium* (Yonge, 1936; Kawaguti, 1950; 1968; 1983; Purchon, 1955; Stasek, 1961; Hartman & Pratt, 1976; Blank, 1986; Jacobs & Jones, 1989; Jones & Jacobs, 1992; Ohno et al., 1995; Persselin, 1998; Vermeij, 2013). These bivalves exhibit specific characteristics of soft body but also microstructural and macroscopic adaptations in shell morphology. Tridacnidea have very large and thick shells, others exhibit semitransparent shells; all adaptations should improve the exposure of the mantle to sunlight (ref. above).

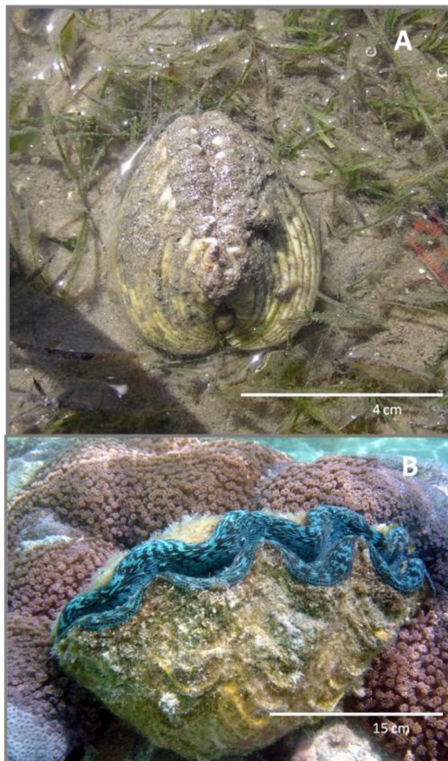

Fig. 2. Two phototrophic bivalves in their natural habitats. A) *Fragum unedo*– mud flat of North Stradbroke Island (QLD, Australia) and B) *Tridacna maxima* (Heterodonta: Veneroida: Cardiidae) – coral reef of One Tree Island (QLD, Australia).

2.3.2 Symbiont-bearing invertebrates in earth's history

Ancient shelled mollusks have a rich and well-documented fossil record; they are confirmed since the early Cambrian (Goedert & Squires, 1990; Peel, 1991; Gubanov et al., 2004; Vinther & Nielsen, 2005; Skovsted et al., 2007; Kiel & Tyler, 2010). The common method to detect ancient symbioses in some fossil bivalves/mollusks is based on structural features of their shells, e.g. the imprints of the elongated anterior adductor muscle and pallial blood vessels in the shells. In addition, palaeohabitat occupation patterns give important hints for symbiotic life styles (Taylor & Glover, 2000; Amano et al., 2007). Indirect tools to date back symbiotic mollusks evolution (estimated molecular age) are molecular clocks (Baco et al., 1999; Shank et al., 1999; Distel et al., 2000; Kano et al., 2002). Upcoming direct molecular tools are biogeochemical analysis of biosignatures which are described in detail below.

The longest fossil record and hence the oldest supposed symbiotic bivalves are the Lucinidae and Solemyidae. The existence of fossil Solemyidae dates back to the Ordovician (Kiel, 2010), Lucinidae first appear into Silurian (Taylor & Glover, 2006). It was suggested that symbiotic relationship of both groups are ancient (Taylor & Glover, 2000, 2006; Taylor et al., 2008). Thyasirids are possibly much younger; they were first described from the Early Cretaceous and were found at seeps and wood falls, which indicates possible symbiotic lifestyle (Kiel et al., 2008a; Kiel & Dando, 2009). Vesicomyids are also associated with seep deposits from the beginning of their appearance in the Middle Eocene; from the Late Eocene onwards they could be found in large numbers at vents and seeps (Kiel & Tyler, 2010 and references therein). Bathymodiolins also appear in the Middle to Late Eocene (Goedert & Squires, 1990; Squires & Goedert 1991; Taviani, 1994; Kiel & Goedert, 2006a; Kiel & Little, 2006). All of these fossil mollusks had recent relatives, thus an ancient symbiotic lifestyle of their ancestors living in similar environments was deduced (e.g., Goedert & Squires 1990; Taviani, 1994; Goedert & Campbell, 1995; Peckmann et al. 1999, 2002, 2004; Goedert et al., 2003; Majima et al., 2005; Gill et al., 2005; Kiel & Little, 2006; Campbell, 2006; Kiel & Peckmann, 2007).

Inoceramidae disappeared at the end of Cretaceous (Dhondt, 1983) and were first known from the Permian (Cramton, 1988). Some authors also speculated

about chemosynthetic or even photosynthetic lifestyles of some inoceramid species (MacLeod & Hoppe, 1992).

The Cardiidea have a fossil record dating back to the Late Triassic (Keen, 1980; Coan et al., 2000; Morton, 2000; Schneider & Carter, 2001). Recent members of Cardiidea with photosynthetic lifstyle like *Fragum* have a fossil record that dates back to Miocene/Holocene (Keen, 1980), Tridacnids proliferate since the Eocene (Romanek et al., 1987). Furthermore, for the Neogen bivalve Mercenaria "tridacnoides" (Jones et al., 1988), for rudists and some other fossil bivalves a photosymbiotic lifestyle was postulated (Kauffman, 1969; Philip, 1972; Vermeij, 2013).

It was speculated about symbiotic relationships in some extinct non-bivalve species like brachiopods which were associated with chemosynthesis-dominated environments in their fossil record (Sandy, 2010) and even photosymbiosis was postulated for some fossil rostroconchia and brachiopods (Cowen, 1970; 1982; Vermeij, 2013). Fortey (2000) reported that olenid trilobites (Late Cambrian/Ordovician) lived under oxygen-poor and sulfur-rich conditions at the sea floor. Reduced oral structures and extended pleural areas were interpreted as an indication for a symbiotic relationship with sulfur bacteria.

Also fossil members of gastropods inhabiting chemosynthetic ecosystems, e.g. Provannids date back to the Late Cretaceous (Kiel & Tyler, 2010).

Though fossil deep-sea chemotrophic mollusks are relatively well-documented, not much attention is given to the non-seep related shallow water chemo- or phototrophic mollusks. At seep and vent sites the epifaunal mollusks densely colonise the habitat. Detecting fossil endosymbiosis in shallow water mollusks by using biogeochemical techniques has an advantage that possibly various heterotrophic mollusks co-occur in the same substrate. Comparing different species in the same habitat gives a better indication of which might have had symbiotic associations with chemosynthetic bacteria or maybe phototrophic dinoflagellates. In the evolution of bathymodiolid bivalves, it was expected that the ancestors of this modern deep-sea mussels live in shallow water reducing sediments. Thus it is possible that "the first contact" between free-living chemosynthetic bacteria and heterotrophic bathymodiolids did not start in the

deep-sea but in shallower marine environments (Duperron, 2010). It will be really interesting to support these hypotheses by analysis of biosignatures. In shallower water habitats the probability to find fossils of definitively non-symbiotic mollusks among the putative symbiotic ancestor of bathymodiolids is much bigger than at fossil seep and vent deposits. Here, it is possible to compare biosignatures of shell-fossils from different species of the same location, to evaluate their lifestyles (see below).

2.4 Molecular markers in tissue of chemosymbiotic vs. heterotrophic bivalves

Prokaryotes are inhabitants of this planet long before the raise of eukaryotes and metazoans and consequently “invented” most of the biochemical key processes. They are the only organisms capable of primary energy production like chemosynthesis and photosynthesis; fixation of molecular nitrogen is unique to prokaryotes. Thus, all other living organisms are able to perform primary production only with support of their ancient or current endosymbiotic associations with prokaryotes. In any case, metazoans whose major diet is based on their autotrophic symbionts are closer to the bottom of the food chain than metazoans without relationship to such microbes. Some of the symbiotic bacteria in mollusks are located within specialised gill cells, so-called bacteriocytes. In other cases the bacteria are attached extracellularly at the gill tissue (Dubilier et al., 2008; Duperron 2008; Southward, 2008). Fluids, rich in oxygen and sulfide or methane, are drawn into the gill and are absorbed by the bacteriocytes. Furthermore, it was reported that *Calyptogena* uses its foot to dig for sulfide in the sediment and then uses specific transport proteins which transfer sulfide to symbionts in the gill tissue (Zal et al., 2000).

The majority of phototrophic symbionts, the zooxanthellae, are located within mantle tissue, sometimes within the gill filaments of the host bivalve (Yonge, 1981), so that the symbionts are exposed to sunlight.

How may the lifestyle of symbiont bearing mollusks lead to identifiable features or even “patterns”, identifiable in the fossil record?

In case that the major nutrients (carbon, nitrogen and sulfur) of mollusks derive from their prokaryotic symbionts, the host biomass is based on molecules built

by the prokaryotic metabolism. In contrast, heterotrophic mollusks filter out or graze off particulate organic matter from their surrounding environment. Thus, search for fingerprints specific for either heterotrophic or chemo- and phototrophic mollusks must consider autotrophic and/or nitrogen metabolism of the symbionts.

2.4.1 Carbon fixation

Apart from the cellulose-degraders in Teredinidae and methanotrophs, all prokaryotic symbionts fix inorganic carbon autotrophically. The common pathway for CO_2 fixation in chemo- as well as in phototrophic symbionts is the Calvin–Benson cycle (Herry & Le Pennec, 1989; Duperron & Fiala-Médioni, 2007; Dreier et al., 2012). The key enzyme of this pathway is ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO). RubisCO catalyses the fixation of $^{12}CO_2$ slightly faster than fixation of $^{13}CO_2$ (Purich & Allison, 2000). This selection leads to an enrichment of ^{12}C in the biomass relative to ^{13}C (negative $\delta^{13}C$ value). The host obtains the organic carbon from its symbionts (Fiala-Médioni & Felbeck, 1990; Childress & Fisher, 1992), hence $\delta^{13}C$ ratios of host tissue reflect the carbon source. Mentionable is the fact that ^{13}C depletion by chemoautotrophic bacteria using the Calvin–Benson cycle for CO_2 fixation is higher than in photosynthetic algal organisms, because of different specificities of RubisCO form I and II enzymes (Ruby et al., 1987; Blumenberg, 2010; see Table 1). Moreover, these distinct forms of RubisCO have been also described for chemoautotrophs (Robinson & Cavanaugh 1995). They show that form I RubisCO is expressed by the symbionts of *Solemya* and *Bathymodiolus,* exhibiting relatively low $\delta^{13}C$ ratios, whereas form II RubisCO is expressed in the tubeworms *Riftia* and *Tevnia* with higher $\delta^{13}C$ ratios (Childress & Fisher 1992). However, the $\delta^{13}C$ ratios are also influenced by the ratios of source carbon (CO_2) and by translocation of carbon during uptake and transport from symbiont to host (Scott et al., 2004).

Symbionts that oxidise methane are related to type I methanotrophs within the Gammaproteobacteria (Petersen & Dubilier, 2009). Methane serves as electron donor as well as carbon source. Biogenic methane exhibits highly $\delta^{13}C$ depleted signatures (Sugimoto & Wada 1995; Zyakun, 1996). Type I methanotrophs use

the ribulose monophosphate pathway for carbon fixation (Leak et al., 1985) and preferentially consume $^{12}CH_4$ which leads to a further depletion in $\delta^{13}C$ values (Coleman et al., 1981; Grossman et al., 2002).

In summary, $\delta^{13}C$ values of tissue from mollusks which harbor chemoautotrophs and/or methanotrophs (“primary producers”) are all significantly depleted relative to mollusks at higher trophic levels. This depletion patterns should also be expected for tissue of phototrophic mollusks.

Table 1. Some $\delta^{13}C$ values of different chemoautotrophic symbioses and corresponding isotopic discrimination of different carbon fixing pathways (compiled after Roeske & O´Leary, 1984; Brooks et al., 1987; Conway et al., 1989; Fisher, 1990; Kennicutt et al., 1992; Guy et al., 1993; Goericke et al., 1994; Robinson & Cavanaugh, 1995; Cavanaugh & Robinson, 1996; Van Dover et al., 2003; Scott et al., 2004; Van Dover, 2007)

$\delta^{13}C$	Organisms	Carbon assimilation	^{12}C enrichment
-30 ‰ to -34 ‰	Bivalves with chemoautotrophic symbionts	CO_2 to organic C (RubisCO form I)	22 – 30 ‰, (24.4 ‰, form IA)
-8.8 ‰ to -16 ‰	Hydrothermal vent vestimentiferan tubeworms with chemoautotrophic symbionts	CO_2 to organic C (RubisCO form II)	18 – 23 ‰
-18 ‰ to -28 ‰	phytoplankton	CO_2 to organic C (supposed RubisCO form IB)	22 – 30.3 ‰
-39.3 ‰ (thermogenic CH_4) to -76.0 ‰ (biogenic CH_4)	Bivalves with methanotrophic symbionts (in some cases additional chemoautotrophic symbionts)	CH_4 to organic C	5 – 30 ‰

2.4.2 Nitrogen assimilation

The main nitrogen sources of bacterial biomass and hence host tissue in chemoautotrophic symbioses are ammonia (NH_4^+) and nitrate (Johnson et al., 1988; Conway et al., 1992; Lilley et al., 1993; Lee & Childress, 1994; Lee et al., 1999). Ammonia and nitrate are used by bacteria for biosynthesis of amino acids and other nitrogen compounds (Payne, 1973; Reitzer & Magasanik, 1987). Mollusks receive their amino acids from their diet (e.g. Neff, 1972), which is in case of chemosymbiosis mainly based on biomolecules from the symbionts.

Isotopic fractionation of nitrogen may occur during uptake and incorporation of nitrogen by bacterial symbionts (Hoch et al., 1992; Yoneyama et al., 1993; Dreier et al., 2012). Methane-oxidising bacteria, for instance, prefer assimilation of $^{14}NH_3$ (Lee & Childress 1994). Independent of the pathway of nitrogen assimilation, it is known that $\delta^{15}N$ ratio increases by about 3.4‰ per trophic level (Minagawa & Wada, 1984; Peterson & Fry 1987). Accordingly, primary producers must show lower $\delta^{15}N$ in tissue than their consumers (Conway et al., 1989; Conway et al. 1992; Lee & Childress 1994; Colaco et al. 2002; Dreier et al., 2012).

2.4.3 Sulfur oxidation

Thiotrophic endosymbiosis is most common among mollusks (see above). Their energy source is sulfide, which originates from abiogenic reduction of sulfate or from microbial sulfate reduction (Kaplan et al., 1963; Aharon & Fu, 2000; Joye et al., 2004). Sulfide in sediments mostly derives from microbial sulfate reduction; both biogenically and abiogenically generated sulfide is depleted in $\delta^{34}S$ (Kaplan et al., 1963; Kiyosu & Krouse, 1993; Aharon & Fu, 2000; Joye et al., 2004). The pathway of sulfur oxidation does not lead to a significant fractionation of sulfur isotopes. The depleted sulfide from sediment is possibly not just used as an energy source but is also assimilated by sulfide-oxidising symbionts and incorporated in their biomass (Dreier et al., 2012). In contrast, the sulfur compounds of non-thiotrophic mollusks derive from sea-water sulfates with $\delta^{34}S$ ratios being markedly different from that of sulfides in sediments (Kaplan et al., 1963; Trust & Fry, 1992; Michener & Schell, 1994). Thus $\delta^{34}S$

ratios of biomass from thiotrophic mollusks are higher depleted than $\delta^{34}S$ values of non-thiotrophic mollusks (O'Donnell et al., 2003; Mizota & Yamanaka, 2003; Mae et al., 2007; Dreier et al., 2012).

In summary, the isotopic compositions of the biological elements carbon, sulfur and nitrogen in biomolecules from host tissue are excellent biosignatures, providing information about an animal's diet and trophic level (Michener & Schell, 1994; Casey & Post, 2011). However, in order to determine diet of fossil mollusks, preserved biomolecules are needed. Here one may take benefit from the mineralised mollusk shells, which are perfect long term conservation wrappings for organic matter.

2.5 Different biosignatures and stability over geological timescales

Various techniques are used to detect symbiotic prokaryotes in mollusk tissue, such as 16S rDNA sequence analysis, fluorescent *in situ* hybridisation and transmission electron microscopy. After death, soft tissue is degraded and only shells are left. Mollusk shells are mainly composed of calcium carbonate in aragonite and calcite conformation; these crystals are formed between organic matrix layers. Frémy (1855) was the first who described conchiolin, the acid insoluble organic matrix in shells. Later, high proportions of acidic amino acids Asx (Asp+Asn) were found in soluble shell-organics. X-ray/electron diffraction revealed matrix-crystal spatial relations protein structure (β-sheet), and the presence of chitin (Weiner & Traub, 1980; Weiner et al., 1983). Recent studies about shell proteins imply that the organic shell matrix is composed of a macromolecular framework consisting of a chitin-silk fibronin gel with acidic proteins (e.g. Marin & Luquet, 2007; Evens, 2008; Marin et al., 2008). To demonstrate the presence of chitin in shells, staining with the fluorescence dye Calcofluor White may be performed, which binds to cellulose and chitin. Obviously, also the residue of fossil shells could be stained. Maybe a hint for persistence of these biomolecules in the shell matrix.

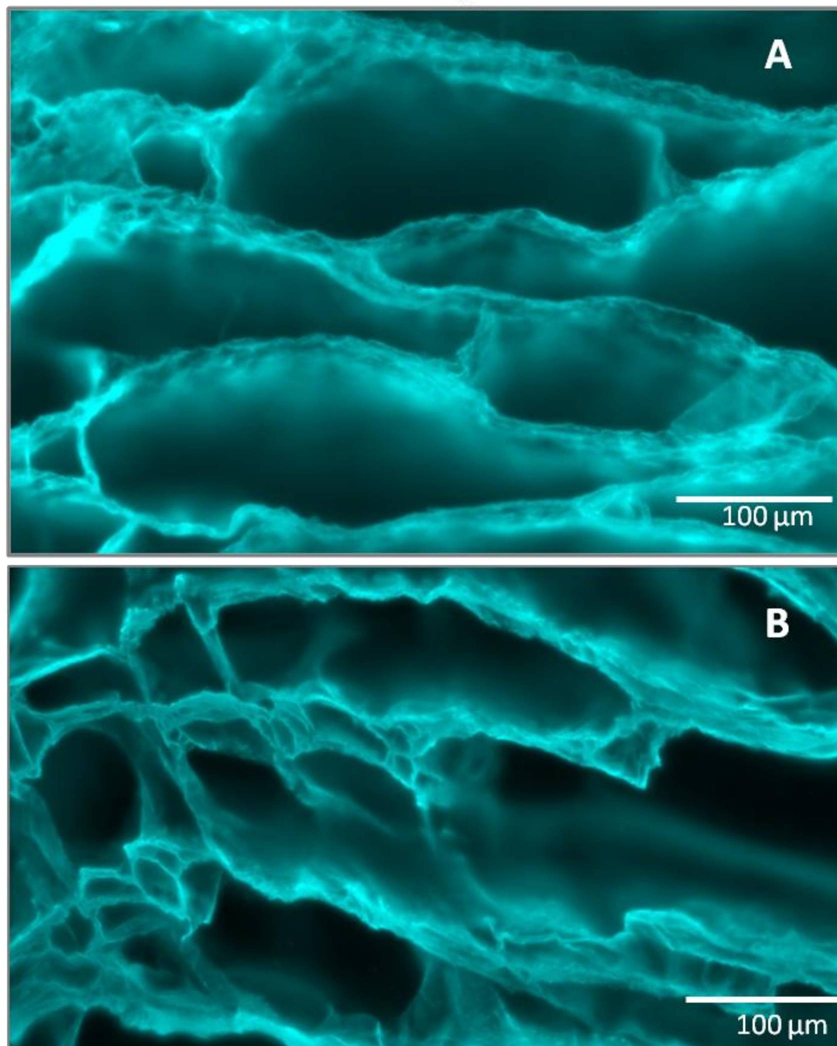

Fig. 3. Chitin staining of a cross section of *Tridacna maxima* decalcified shell.

A) Recent, One Tree Island (QLD, Australien)

B) Pleistocene, north of Dahab (Sinai, Egypt). Cross sections were stained with Calcofluor White.

Figures 3 shows a stained cross section of *Tridacna maxima* shells (recent and fossil) embedded in LR white resin

The cross section was decalcified with 0.5 M EDTA over night and then stained with Calcofluor White. To exclude unspecific binding to embedding resin, an untreated (unfixed, not embedded) piece of *Tridacna maxima* shell was decalcified and stained with Calcofluor White (Fig. 4).

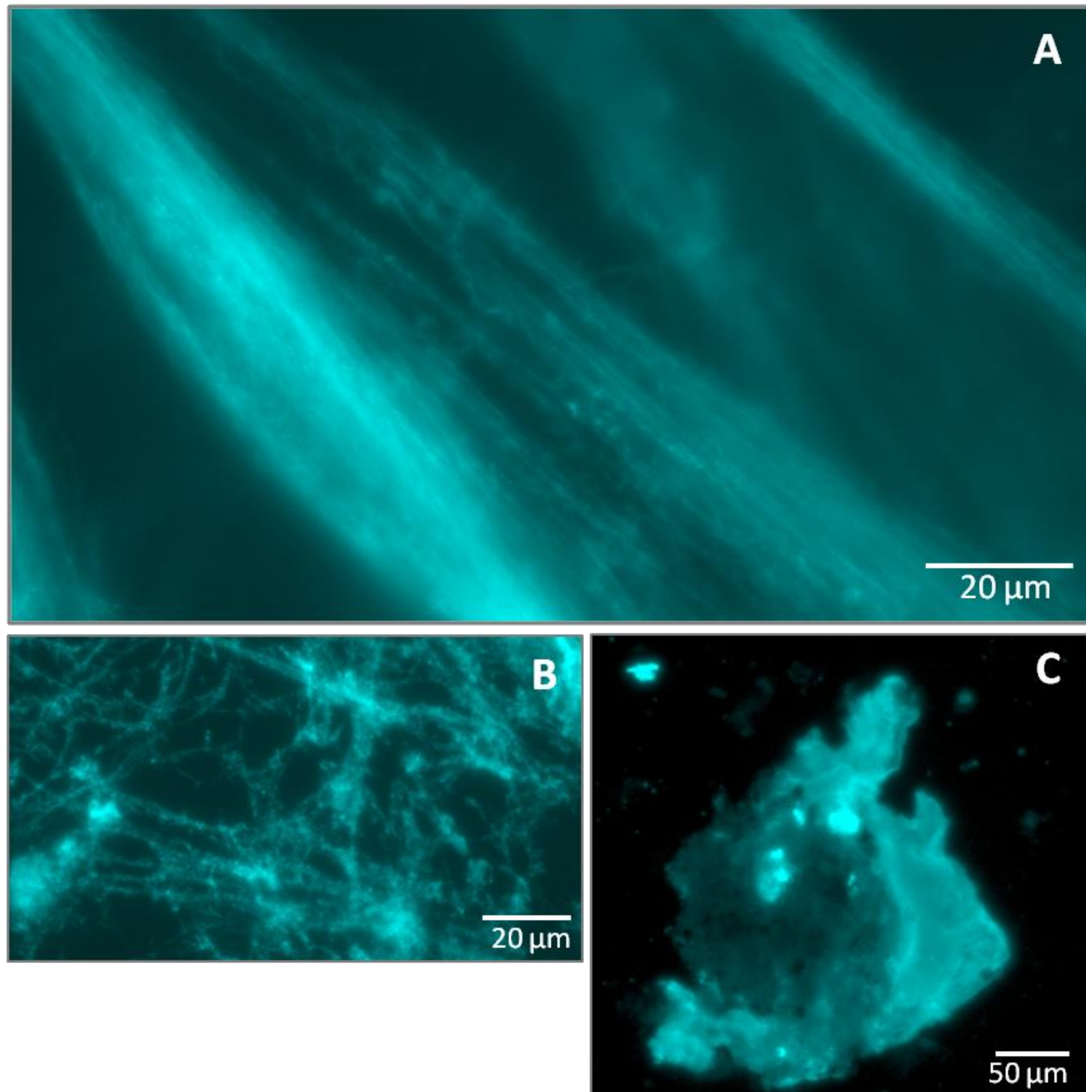

Fig. 4. Chitin staining of decalcified piece of *Tridacna maxima* shell. A), B) Recent, One Tree Island (QLD, Australia) filament like structures are visible. C) Pleistocene, north of Dahab (Sinai, Egypt) no filaments could be detected. Cross sections were stained with Calcofluor White.

It is obvious that the Calcofluor-stained material in the fossil shell of *Tridacna maxima* (Fig. 4 C) is different from the filamentous structure in the recent shell (Fig. 4 A, 4B). In addition, a cross section of a fossil (Upper Cretaceous) *Inoceramus sp.* shell was stained with Calcofluor White. The treatment of cross section equates to that shown in Figure 3 but without decalcification. Figure 5 shows a hint that maybe fossil chitin was stained between the calcium carbonate crystals of the *Inoceramus sp.* shell.

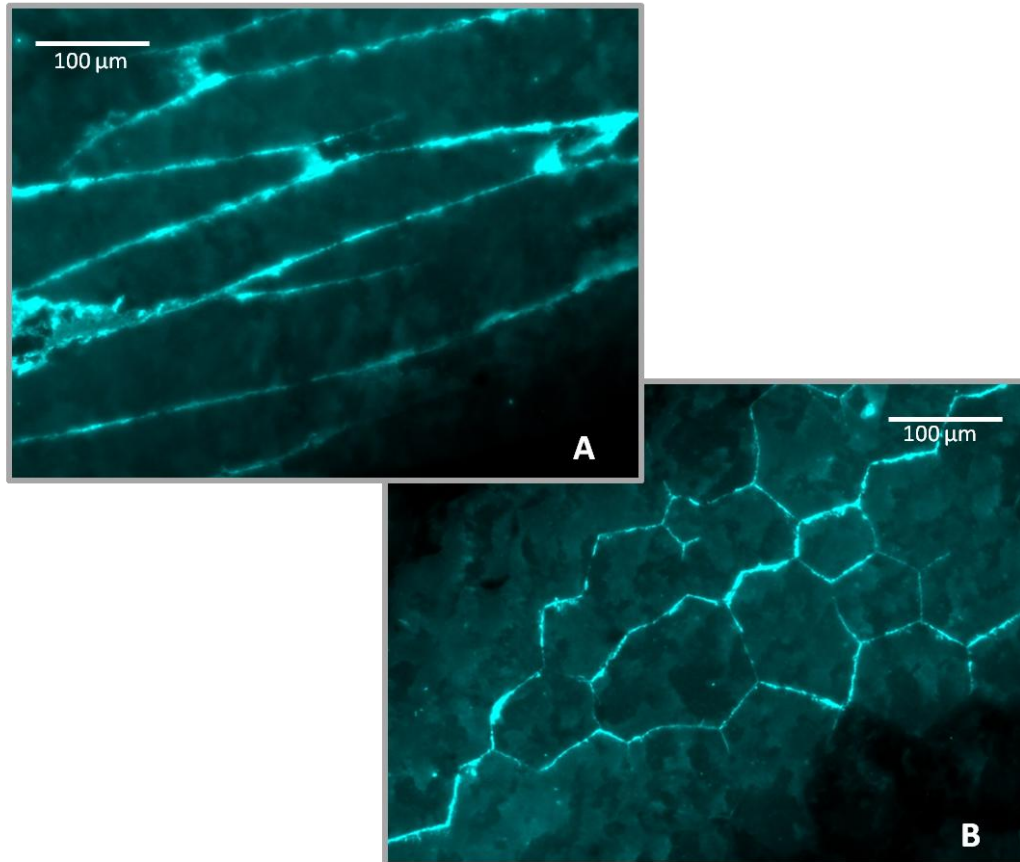

Fig. 5. Chitin staining of a section of fossil shells of *Inoceramus sp.* (Upper Cretaceous, quarry Dammann South, Söhlde, Germany, Heterodonta: Veneroida: Cardiidae). A) Longitudinal section of shell stained with Calcofluor White. B) Cross section of the same shell.

The preserved biomolecules of the shell will provide information about the mollusk's diet. In endosymbiont-bearing mollusks, carbon, nitrogen and sulfur are taken up by the symbionts, get an isotopic fingerprint and are then incorporated in mollusk biopolymers (see above). Since the remains of the organic shell matrix are preserved after death, stable isotope analysis of the matrix serves as valuable screening tool for detecting symbiotic association in living as well as in fossil mollusks.

In many studies $\delta^{13}C$, $\delta^{15}N$ and sometimes $\delta^{34}S$ in soft tissue were determined in order to analyze dietary intake (Kennicutt et al., 1992; Dando & Spiro, 1993; Conway et al., 1994; Dando et al., 1994; Fischer, 1995; Colaco et al., 2002; Dattagupta et al., 2004; Lorrain et al., 2002; Carlier et al., 2007; 2009). However, only few studies describe these isotopic fingerprints with respect to the organic matrix of recent and fossil shells (O'Donnell et al., 2003; Mae et al.,

2007; Dreier et al., 2012). Only the study by Dreier et al. provides $\delta^{34}S$ values of the organic matrix of empty shells from recent bivalves and subfossil (Late Pleistocene) shells.

It was shown that sulfur isotopes are not useful markers to detect ancien thiotrophic lifestyle, because after death of the mollusks $\delta^{34}S$ values in the organic matrix will decrease. It was assumed that the reason could be the instability of sulfur-containing amino acids (Jones & Vallentyne, 1960) or sulfides derived from proteolysis and from bacterial sulfate reduction during soft tissue degradation. New results confirm the latter hypothesis: the non-symbiotic bivalve *Venerupis aurea,* which was used in the study of Dreier et al. (2012), was degraded in original sediments under laboratory conditions in an aquarium. After half a year the shells were analyzed and $\delta^{34}S$ as well as C/N ratios were measured. The C/N ratio is an expression for the grade of alteration and decay of the organic shell matrix (Ambrose, 1994). The C/N ratio of the artificial degraded shells of *Venerupis* slightly increased from 3.15 (fresh shell) to 3.27 (degraded half a year), the $\delta^{34}S$ ratio dropped slightly from 7.8‰ to 7.5‰. Longer resting time in the sediment is needed to futher decrease the $\delta^{34}S$ ratio further (Dreier et al. 2012).

The enrichment of sulfides during degradation of soft tissue may be the source of the framboidal pyrite formation (Fig. 6; Berner, 1984; Wilkin, 1995) and for the decreasing $\delta^{34}S$ ratio of shells after death.

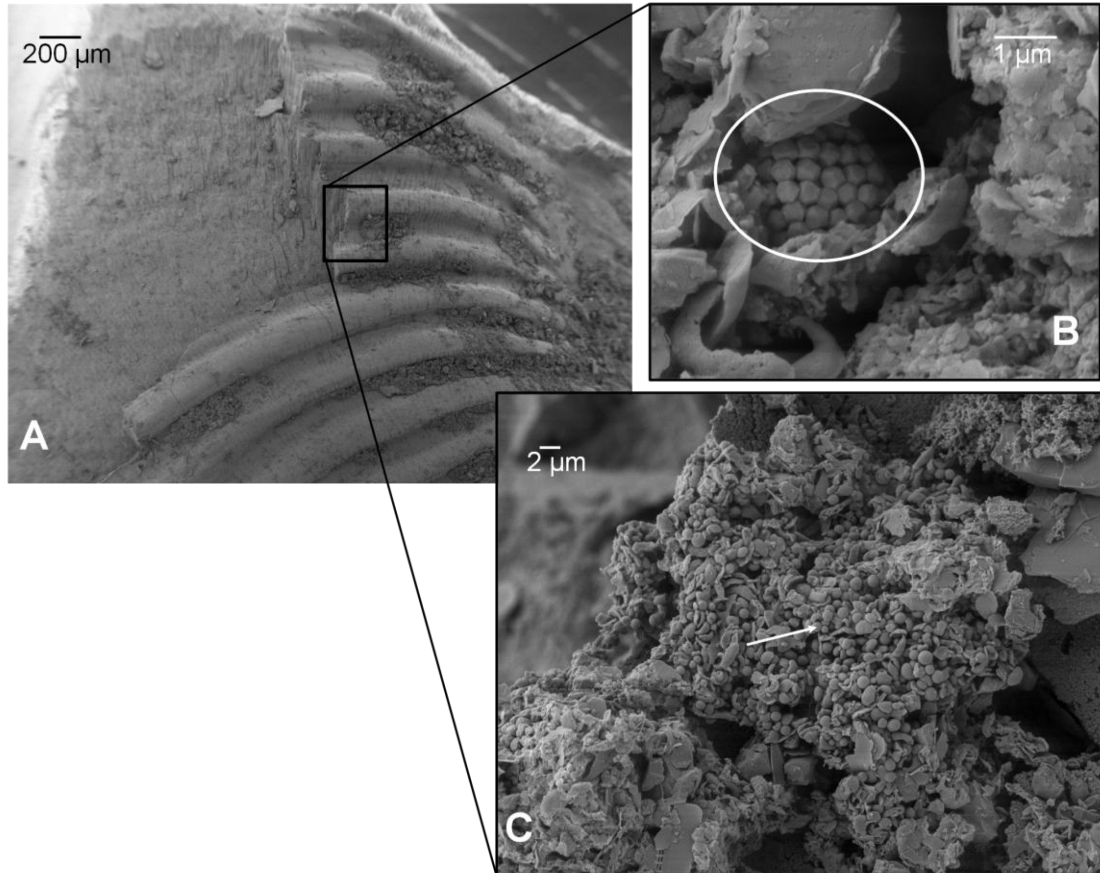

Fig. 6. Scanning electron micrographs of a *Venerupis* shell (Heterodonta: Veneroidae) after 6 months incubation in sediment. (A) general view of the shell. (B) higher magnification, with framboidal pyrite (circle). (C) higher magnification of left picture, with bacteria attached to the shell surface [arrow].

Lipids are another prominent group of biochemical markers, which are analyzed to identify symbiosis in mollusks. Fatty acids as main building blocks of lipids have a characteristic distribution pattern. Short-chained monounsaturated fatty acids (MUFA) are of mainly prokaryotic origin (Bishop, 1976) whereas the major component of eukaryotic lipids consist of long-chained polyunsaturated fatty acids (PUFA; Shaw, 1974). Their specificity and structural diversity make them to important trophic biomarkers in marine ecology (Gehron & White, 1982; Parkes & Taylor, 1983; Guckert et al., 1985; Sargent et al., 1987; Wakeham & Canuel, 1988; Findlay et al., 1990; Sargent et al., 1990; Bradshaw et al., 1991; Hopkins et al., 1993; Rajendran et al., 1993).

Lipids were also used to characterise symbiotic associations between prokaryotes and marine invertebrates (Berg et al., 1985; Conway & Capuzzo,

1990, 1991; Ben-Mlih et al., 1992; Zhukova et al., 1992; Cobabe & Pratt, 1995; Fullarton et al., 1995). In mollusks the lipid content depends on dietary lipid intake (Moreno et al. 1980; Piretti et al. 1987), thus lipid content of mollusks with autotrophic symbionts will reflect a diet based on the symbionts. It is known from bivalve shells that they contain lipids like fatty acids, cholesterol, phytandienes, ketones and sometimes *n*-alkanes. Lipids are geologically stable which make them well-suited for paleontological approaches. In addition, lipids have low solubility in water at low temperatures; hence in early diagenesis the level of contamination from surrounding pore fluids and the migration of lipids out of the shell is low. As mentioned above, the carbon of symbiont-derived compounds is depleted in $\delta^{13}C$, furthermore it is known that lipid carbon was found to be depleted by 3‰ relative to their dietary carbon (DeNiro & Epstein, 1977; Crenshaw, 1980). Consequently, $\delta^{13}C$ ratios of most mollusks shell-lipids may reflect if they are symbiont-bearing or not. Cobabe & Pratt (1995), Conway & Capuzzo (1991) and Dreier et al. (2012) found some fatty acids of chemotropic bivalves to be more depleted in $\delta^{13}C$ relative to heterotrophic bivalves. Lipids from fossil shells of two bivalve species about 1.4 million years old show a fatty acid distribution very similar to modern shells (with differences in their relative abundance; Cobabe & Pratt, 1995). However, $\delta^{13}C$ values of fossil shell-lipids have been not reported so far.

2.6 Future perspectives

In the light of recent climatic and global changes it will be more and more important to reconstruct environmental conditions of the past. Especially the marine environment represents an important climatic driving force and changing conditions could be recognised by a change in the benthic ecosystem. Today the stability of many ecosystems is in danger, also because of the breakdown of symbiotic interactions, just considering e.g. bleaching events in coral reefs (Carpenter et al., 2008).

Chemosymbiotic species are major players at oxic-anoxic interfaces of the sediment or at seep and vent sites, for example at sites of methane-hydrate breakdown. The influence and importance of chemosymbiotic species at places

with high eutrophication, leading to anoxic events, is not well-understood, though one may expect that eutrophication also leads to mass development of chemosymbionts (Hesselbo et al., 2000).

If we even could identify the point where the lifestyle of a species switches from heterotrophic to symbiotic, we will be also able to find factors driving emergence of cooperative microbial-host associations, which will foster our understanding of this evolutionary driving force. Mollusks are very suitable model organisms, because they have a well-documented fossil history and provided mineralised tissue.

In some cases original organic matrix is preserved in fossil shells. By analyzing the isotopic composition of the remaining original organic matrix and of separately extracted lipids of fossil mollusks shells, it is possible to distinguish between "primary consumers" (chemo -and phototrophic) and mollusks from higher trophic levels. In order to get trustworthy data it is recommendable to analyze at least two species with different diets from the same habitat or location, otherwise the reliability of isotopic dates are questionable (Dreier et al., 2012). For instance, Dreier et al. (2012) found $\delta^{13}C$ and $\delta^{15}N$ values for the heterotrophic bivalve *Venerupis (*$\delta^{13}C$ of -24.1‰ and $\delta^{15}N$ of +4.2‰) in the same range as for chemotrophic bivalves from other sites. But in contrast, compared to the values of the chemotrophic bivalves from the same site, the large differences between the isotopic signatures allowed to distinguish between the two lifestyles. With this respect it is also important to keep in mind that some diets of endosymbiotic mollusks are not completely based on their symbionts. Some of the mollusks still use filter-feeding as an additional option (Duplessis et al., 2004). To date no isotopic data are available for chemo-compared with phototrophic mollusks inhabiting the same site, so is not known if there is a resolution limit between the two different primary producers' lifestyles.

Generally it should be possible to confirm either autotrophy or heterotrophy by comparing carbon and nitrogen isotopies of the organic matrices from different candidate shell specimens from the same location. This method is not limited to mollusks – all invertebrates with mineralised tissue and embedded organic

matrix could be analyzed, for example also shells of brachiopods and perhaps even organic matrices of tubeworm tubes.

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3. The fingerprint of chemosymbiosis: origin and preservation of isotopic biosignatures in the nonseep bivalve *Loripes lacteus* compared with *Venerupis aurea*

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3.1 Abstract

Endosymbionts in marine bivalves leave characteristic biosignatures in their host organisms. Two non-seep bivalve species collected in Mediterranean lagoons, thiotrophic symbiotic *Loripes lacteus* and filter-feeding non-symbiotic *Venerupis aurea* were studied in detail with respect to generation and presence of such signatures in the living animals, and the preservation of these signals in subfossil (late Pleistocene) sedimentary shells. Three key enzymes from sulfur oxidation (APS-reductase), CO_2 fixation (RubisCO) and assimilation of nitrogen [glutamine synthetase (GS)] were detected by immunofluorescence in the bacterial symbionts of *Loripes*. In *Loripes,* major activity derived from glutamine synthetase of the symbionts whereas in *Venerupis* the host GS is active. In search of geologically stable biosignatures for thiotrophic chemosymbiosis that might be suitable to detect such associations in ancient bivalves, we analyzed

the isotopic composition of some shell-lipids ($\delta^{13}C$) and the bulk organic matrix of the shell ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$). In the thiotrophic *Loripes,* $\delta^{13}C$ values were depleted compared to the filter-feeding *Venerupis* by as much as 8.5 ‰ for individual fatty acids, and 4.4 ‰ for bulk organic carbon. Likewise, bulk $\delta^{15}N$ and $\delta^{34}S$ values were more depleted in recent thiotrophic *Loripes*. Whereas $\delta^{34}S$ values were found to be unstable over time, the combined $\delta^{15}N$ and $\delta^{13}C$ values in organic shell extracts revealed a specific signature for chemosymbiosis in recent and subfossil specimens.

3.2 Introduction

Thiotrophic endosymbiosis is a widespread lifestyle in marine habitats. Endosymbiotic associations occur at the oxic-anoxic interface of hydrothermal vents, cold seeps, reducing sediments, wood and whale falls in the deep sea as well as in shallow water habitats providing dysoxic conditions (Lonsdale, 1977; Corliss et al., 1979; Jannasch and Wirsen, 1979; Van Dover, 2000; Treude et al., 2009; Kiel and Tyler, 2010).

Animal hosts bearing sulfur-oxidizing symbionts are known from five recent animal phyla (Ciliophora, Platyhelminthes, Nematoda, Annelida and Molluska; Dubilier et al., 2008 and references therein). Despite the high phylogenetic diversity of the hosts, endosymbiotic microorganisms appear to be less diverse, with nearly all thiotrophs belong to the group of *Gammaproteobacteria* (Cavanaugh, 1994; Dubilier et al., 2008).

Chemosymbiosis between mollusks and sulfur-oxidizing bacteria occur in seven bivalve families, Lucinidae being the most diverse (Taylor & Glover, 2009; 2010; Taviani, 2011, and references therein; Taylor et al., 2011). All known recent members of the Lucinidae harbor thiotrophic bacteria within their gill tissues. The large gills constitute approximately half of the weight of the entire soft tissue.

While endosymbiotic invertebrates at cold seeps and hot vents are relatively well studied (e.g. Childress & Fisher, 1992; Conway et al., 1994), knowledge about those living in more widespread marine settings is scarce. Here, we focus on the infaunal, nonseep bivalve *Loripes lacteus* that is common in shallow

water habitats characterized by reducing sediments (Johnson et al., 2002). The genus *Loripes* is almost cosmopolitan in distribution and well documented in the Cenozoic record (Heinberg, 1999; Monegatti & Raffi, 2001; Schneider et al., 2005). Our study was aimed at identifying specific biosignatures of nonseep-related thiotrophic endosymbiosis in *Loripes*, with the ultimate goal of establishing a tool to reconstruct the lifestyle of fossil bivalves.

Generally, the isotopic compositions of the biological elements carbon, sulfur and nitrogen in biomolecules are excellent biosignatures providing information about an animal's diet (Michener & Schell, 1994). For instance, it is well known that the carbon isotopic composition of tissue from a heterotrophic animal reflects the $\delta^{13}C$ of its diet (DeNiro & Epstein, 1978). As in all other members in the family Lucinidae, the symbiosis in *Loripes* is based on the oxidation of sulfide. In marine sediments, the majority of the sulfide originates from microbial sulfate reduction (Kaplan et al., 1963; Aharon & Fu, 2000; Joy et al., 2004). Like in filter-feeding bivalves, the sulfide-rich water is drawn in through a siphon and transported into the branchial chamber where sulfide and oxygen are taken up by the gill tissue and transferred to the symbionts. In free-living bacteria, different pathways for the dissimilatory oxidation of sulfide have been described (e.g. Brüser et al., 2000). Some aerobic chemolithotrophs oxidize reduced sulfur directly to sulfate by the *Sox* system (Rother et al., 2001; Friedrich et al., 2005). In the two other pathways the first oxidation product of HS^-, $S_2O_3^{2-}$ or S^0 is sulfite. The further oxidation to sulfate could be mediated either by the sulfite oxidase, which is the most widespread system, or by the reverse-operating enzymes of the sulfate-reduction process (Meyer & Kuever 2007). A diagnostic enzyme of the latter pathway is a reverse operating homologue of the adenosine phosphosulfate (APS) reductase. The APS reductase catalyzes the binding of sulfite to AMP and APS is generated. In contrast, the sulfite oxidase pathway oxidizes sulfite directly to sulfate. It was shown, for example, that the endosymbiont of the tube worm *Riftia pachyptila* oxidizes sulfide via reverse sulfate reduction (Markert et al., 2007) and there is evidence that the endosymbionts of lucinid bivalves also use this sulfur oxidation pathway (Herry & Le Pennec, 1989; Duperron & Fiala-Médioni, 2007). Reverse sulfate reduction does not fractionate the sulfur isotopes (Brunner & bernasconi, 2005

and references therein), but the sulfide that is taken up by the bivalve from the sediment was depleted in $\delta^{34}S$ (Kaplan et al., 1963). Thus $\delta^{34}S$ of biomass may be a potential biosignature of thiotrophic endosymbiotic animals.

The symbionts of *Loripes* fix CO_2 via the Calvin-Benson cycle (Herry et al., 1989; Duperron & Fiala-Médioni, 2007) whose key enzyme is the ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO). The fixation of $^{12}CO_2$ is catalyzed more rapidly by the RubisCO than that of $^{13}CO_2$ (Purich & Allison 2000) and thus the ^{12}C isotope is enriched in the biomass relative to the ^{13}C isotope (negative $\delta^{13}C$ value). Moreover, ^{13}C depletion by chemoautotrophic bacteria using the Calvin-Benson cycle for CO_2 fixation is higher than in photosynthetic algal organisms, because of different specificities of RubisCO enzymes (Ruby et al., 1987; Blumenberg, 2010). Consequently, the differences in $\delta^{13}C$ ratios reflect the carbon source of the bivalves (either endosymbiont biomass or water-born organic matter from photosynthetic primary production). Bivalves that obtain organic carbon from chemoautotrophic endosymbionts were found to have $\delta^{13}C$ values ranging from -27 ‰ to -35 ‰ (Childress & Fisher 1992; Robinson & Cavanaugh 1995; Cavanaugh & Robinson 1996). These isotopic patterns have been described for lipids and proteins (Conway & Capuzzo, 1991; O´Donnell et al., 2003). As all protein amino acids originate from the diet of the bivalves (Neff, 1972) and protein serves as a matrix component for the shell mineral phase (Crenshaw, 1980), the isotopic pattern could also be observed in the shell.

The nitrogen source for bacterial and host biomass may vary as well. The main sources of inorganic nitrogen for chemoautotrophic symbioses are ammonia (in most cases as ammonium ion NH_4^+) and nitrate (Johnson et al., 1988; Conway et al., 1992; Lilley et al., 1993). Bacterial assimilatory nitrate reductase has been detected in some but not in all chemosymbiontic bivalves tested so far (Lee & Childress, 1994). GS is the key enzyme for assimilation of ammonium to glutamine (Stewart et al., 1980; Reitzer & Magasanik, 1987), and is also relevant for nitrogen uptake, in particular for symbiotic organisms living in reducing sediments (Lee & Childess, 1994; Lee et al., 1999). Bacterial glutamine synthetase (GSI) consists of 12 identical protein subunits (Brown et al., 1994) and is stable after moderate heating, whereas the eukaryotic

glutamine synthetase (GSII) is an octameric protein, which is thermally unstable (Merrick & Edwards, 1995). The reaction catalyzed by GS fractionates ^{15}N isotopes. For a GS isolated from chloroplasts, a depletion of $\delta^{15}N$ by -16.5 ±1.5 ‰ was observed *in vitro* (Yoneyama et al., 1993). In bacteria, the uptake of ammonium across the membrane leads to additional ^{15}N depletion (Hoch et al., 1992), which will be also reflected in ^{15}N-depletion of the host's biomass.

Numerous studies used stable isotopes of tissue to analyze dietary intake and environmental situations of vent and seep organisms (Kennicutt et al. 1992; Conway et al. 1994; Fisher, 1995; Colaco et al., 2002; Dattagupta et al., 2004). But as yet, only few studies used the isotopic approach to investigate nonseep-related fauna (Dando & Spiro, 1993; Dando et al., 1994; Lorrain et al., 2002; Carlier et al., 2007; 2009). Most of these approaches focused on food web structures and nutrient input and exclusively analyzed soft tissue samples. In one of the few data sets originating from modern and fossil shell, $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values from the infaunal bivalve *Mercenaria* spp. were used as indicators for a change in diet sources over time (O´Donnell et al., 2003). In our current study, we adopted the same approach to distinguish between heterotrophic and chemosynthetic lifestyles of bivalves.

Little is known about endosymbiosis in ancient non-seep settings and hence the evolution and ecological importance of such associations in Earth's history. A few studies have attempted to determine whether the diet of bivalve fossils was based on chemosymbiosis or filter feeding (e.g. CoBabe & Pratt, 1995; Mae, et al., 2007). However, these studies were based on only one biosignature (lipids or protein matrix) and did not include information about the expressed metabolic pathways of host and symbiont. Our study will combine relevant information on the expression and activity of key enzymes with isotopic data from modern to subfossil (Pleistocene) nonseep bivalves with the aim to differentiate between heterotrophic and thiotrophic lifestyles of recent and ancient bivalves.

3.3 Materials and methods

3.3.1 Sample collection and fixation

Loripes lacteus and *Venerupis aurea* (*Gastrana fragilis,* only from sampling site VeniceB; see below) were obtained from sediment of the lagoonal Ganzirri Lake (Sicily, Italy, 38°15'36''N, 15°36'55''E, about 1 m depth) in June 2010 and the Venice lagoon (Italy) in April 2011 (VeniceA: San Leonardo 45°20'11"N, 12°14'30"E, about 2 m depth) and in July 2011 (VeniceC: Sant'Andrea, 45°26'13''N, 12°23'01''E about 50 cm depth; VeniceB: Ottagono Alberoni, 45°20'55.8''N, 12°17'38.8''E about 2 m depth). All *Loripes lacteus* specimens from the Venice Lagoon were sampled on meadows of the seagrass *Zostera marina*.

Subfossil (late Pleistocene, original shell mineral not recrystallized) *L. lacteus* and *V. aurea* are provided by ISMAR-CNR (Bologna) and were collected aboard the RV Urania during ARCO cruise 2008 (41°59'39.6"N, 16°15'4.2"E). For biomarker and DNA extraction the samples were kept frozen at -15°C until dissection in the lab. For immunofluorescence analysis the gill tissue was fixed overnight at 4°C in 4 % paraformaldehyde in 0.1 M phosphate-buffered saline pH 7.4 (PBS) containing 0.9 % (w/v) NaCl and 10 % (w/v) sucrose. After fixation tissue was washed two times for 15 min with 0.1 M phosphate-buffered saline (PBS) containing 0.9 % NaCl, 10 % sucrose and 10 mM glycine. The samples were then dehydrated in an ascending series of ethanol concentrations and then stored at 4°C.

3.3.2 16S rRNA gene analysis

The gill tissue of two to four individuals of sampled *L. lacteus* were homogenized with a mortar and pestle in liquid nitrogen. Genomic DNA was extracted from homogenate using the DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany). The 16S rRNA genes were amplified as described (Wrede et al., 2011) using 8f (5′-AGA GTT TGA TCA TGG CTC AG-3′) and 1492r (5′-GTT ACC TTG TTA CGA CTT-3′) (Alain, et al., 2006) as primers. The PCR-products serve as template for the following nested PCR using a mix of 907Rc and 907Ra as reverse and 341f-GC as forward primers (Schäfer et al., 2001). The fragments from the nested PCR were separated by denaturing gradient gel

electrophoresis (DGGE, Muyzer et al., 1993; Wrede et al., 2011). Excised from the gel and purified as described, amplification products were sequenced by the Göttingen Genomics Laboratory (Göttingen, Germany).

3.3.3 Immunofluorescence

Pieces (2 mm^3) of the dehydrated gill tissue were embedded in Technovit 7100 (Heraeus Kulzer GmbH) and thin sectioned (1 µm thickness) with an Ultracut E Ultramicrotome (Reichert, Vienna, Austria) using glass knives (cf. Wrede et al., 2008). For labelling, sections were placed on gelantine/chromalaun coated glass slides (Nussbaumer et al., 2006); in all further steps, solutions were directly applied to the sections and removed after incubation with filter paper. First, sections were incubated in 3 % (w/v) bovine serum albumin (BSA, in 50 mM potassium phosphate buffer containing 0.9 % [w/v] sodium [PBS]) for 30 min, followed by incubation in 10 % (v/v) goat preimmune serum in 50 mM PBS for 20 min. Then the primary antibody directed against APS reductase (rabbit anti-AprB antibodies; Eurogentec, Seraing, Belgium), RubisCO (anti-RbcLI antibodies; Agrisera, Vännäs, Sweden) or GSI [anti-glutamine synthetase (*E. coli*); abcam, Cambridge UK] was applied and incubated for 2 h. Dilutions of 1:50 of original antiserum were used for the labeling. Unbound antibodies were removed by washing sections three times for 5 min with 50 mM PBS containing 0.05 % (w/v) Tween20 and one time for 5 min with 50 mM PBS. Then the secondary antibody [dilution 1:250, Alexa Fluor®546 goat anti-rabbit IgG (H+L) or FITC-rabbit anti-goat IgG; Invitrogen, Carlsbad, CA, USA] were applied and incubated for 1 h. The washing step was repeated, followed by two washing steps for 10 s with double-distilled water. Immunofluorescence images were taken with a Zeiss Axioskop 40 microscope equipped with an Axiocam MRm camera (Carl Zeiss, Göttingen, Germany). For Alexa Fluor®546 and FITC coupled antibodies filter set 43 (Carl Zeiss, Göttingen, Germany) and filter set YFP HC (AHF Analysetechnik, Tübingen, Germany) were used, respectively. Semithin (1 µm) sections of embedded gill tissue were also used for fluorescence *in situ* hybridization (FISH) with CY3-labelled EUB338 I-II probe (Biomers, Ulm, Germany) as described previously (Perntaler et al., 2001).

3.3.4 Detection of GSI by Western blot

Western blotting was performed as essentially described by Towbin et al. (1979). Tissue protein extracts were prepared as for enzyme activity analysis (see below). Extracts were denatured in buffer containing 0.14 M Tris-HCl, 4 % SDS, 26.1 % (v/v) glycerol, 16 % (v/v) beta-mercaptoethanol and 0.01 % Bromophenol Blue, pH 7.0 by heating at 95°C for 10 min. Samples were loaded on 12 % (w/v) acrylamide gels. After denaturing gel electrophoresis, proteins were transferred on a PVDF membrane (Applichem Inc., Darmstadt, Germany) with a semi-dry blotting apparatus (Biometra, Göttingen, Germany) for 1 h at 90 mV. The membranes were incubated with polyclonal anti-GS (*E. coli*) antiserum raised in goat (abcam) and detected by an alkaline-phosphatase-conjugated secondary antibody system (Promega, Madison, WI).

3.3.5 Enzyme activity assays

The activities of bacterial GSI and eukaryotic GSII were measured in protein extracts from gill tissue and complete soft tissue *of L. lacteus* and *V. aurea* (from VeniceC). As positive control for GSII, activity was measured in protein extracts from mouse liver and kidney as positive control for GSI, activity from GS of *E. coli* (Sigma-Aldrich, Deisenhofen, Germany) was measured. Frozen tissue samples were homogenized in liquid nitrogen with a porcelain mortar and pestle in ice-cold buffer containing 200 mM NaCl, 50 mM Tris-HCl at pH 7.5. Homogenates were sonicated three times for 10 s, and then centrifuged at 13000 rpm for 30 min at 4°C. Supernatants were used for activity assays. Protein concentration in extracts was determined according to Bradford (1976).

For measurement of GS activity, the γ-glutamyltransferase assay was performed according to the method of Bender et al. (1977). A reaction mixture contained 135 mM imidazole-hydrochloride, 18 mM hydroxylamine-hydrochloride, 0.27 mM $MnCl_2$, 25 mM potassium arsenate, 0.36 mM sodium ADP and 20 mM L-glutamine, pH 7.55 at 37°C. The reaction was started by addition of protein extracts. To terminate the reaction, a mixture of 0.2 mM $FeCl_3$, 0.12 M trichloracetic acid and 0.25 M HCl was added. The absorbance was measured photometrically at a wavelength of 540 nm. A blank was prepared in the same way, but arsenate and ADP solutions were replaced by

distilled water. For inactivation of GSII protein extracts were heated for 10 min at 60°C.

3.3.6 Lipid extraction and isotopic analysis

Shells from two and three individuals of recent (living) *Loripes* and *Venerupis* were cleaned under a binocular, using needle, scalpel, brass brush and sterile double-distilled water to remove organic and mineral contamination from the shell surface. After cleaning, shells were washed two times in sterile double-distilled water. Then the shells were pulverized using a porcelain mortar and pestle. Gill tissue was also homogenized using a porcelain mortar and pestle. Homogenates were subjected to an alkaline hydrolysis with 6 % KOH in methanol for 2 h at 70°C by successive sonication, followed by the extraction of neutral lipids with *n*-hexane for three times. The remaining solution was acidified to pH 1-2 and again extracted three times with *n*-hexane. The latter combined extracts were converted with trimethylchlorosilane/MeOH 90 min at 80°C to methyl esters. The extract of neutral lipids was silylated with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for 90 min at 80°C and dissolved in *n*-hexane with activated copper chips (to remove elemental sulfur). Compounds were identified via GC-MS analysis (Varian CP-3800 chromatograph coupled to a 1200L mass spectrometer, Agilent Sta. Clara, CA, USA) after comparison with published data. $\delta^{13}C$ values of selected lipids were measured using a Trace GC gas chromatograph coupled to a Delta Plus isotope-ratio mass spectrometer (both Thermo Scientific, Waltham, MA, USA). The combustion reactor contained CuO, Ni, and Pt and was operated at 940°C. The stable carbon isotope compositions are reported in the delta notation ($\delta^{13}C$) vs. the Vienna Pee Dee belemnite (V-PDB) standard.

3.3.7 Isotopic analysis of bulk organic matter

Modern (living) and subfossil shells taken from 8 to 16 individuals were cleaned and homogenized as described for extraction of lipids (see above). The shell powder was placed into dialyze tubes (Serva, Heidelberg, Germany) and then dialyzed (Mae et al., 2007) against sterile 0.5 M eEDTA (pH 7.4; Carl Roth GmbH, Karlsruhe Germany) at room temperature. EDTA solution was changed

every two days. After 2-3 weeks, the mineral phase was dissolved and the remaining organic matrix appeared as brown to yellow flakes. Then the samples were exhaustively dialyzed against sterile double-distilled water. Finally, the organic matrix was freeze-dried. The dry bulk organic matrix was used for $\delta^{13}C$-, $\delta^{15}N$- and $\delta^{34}S$ isotopic measurements. Measurement was performed via Vario EL III GVI Isoprime isotope-ratio MS (Sieper et al., 2006) at the Laboratorium für Stabil-Isotopenanalytik (Schweitenkirchen, Germany).

3.4 Results

3.4.1 Identification of symbionts by 16S rRNA gene analysis

The presence of symbionts in *L. lacteus* was verified by FISH and analysis of the amplified 16S rRNA gene sequences from gill tissue. In fact, a 16S rRNA gene sequence displaying 99 % similarity to a sequence from *L. lacteus* gill symbionts (accession no. FJ752447) was found. No sequences of methylotrophic bacteria were retrieved. In addition, sequences matching to the cyanobacterium *Synechococcus sp.* (accession no. DQ023295.1) were amplified from gill tissues of *Loripes* and *Venerupis*. The latter served as a symbiont-free bivalve in control experiments.

3.4.2 Carbon dioxide fixation and related $\delta^{13}C$ values

The symbiont cells were located in gill tissue cells of *L. lacteus* (Fig 1). Carbon dioxide fixation via Calvin-Benson cycle was confirmed by detection of the key enzyme RubisCO. Figure 2a shows single endosymbiont cells displaying a strong fluorescence signal indicating bound RubisCO antibodies.

With regard to verifying this pathway of carbon dioxide fixation in fossil bivalves, we determined the respective $\delta^{13}C$ fingerprint of some lipids and of bulk organic shell matrix taken from the shells of living and subfossil *L. lacteus*. The $\delta^{13}C$ values of lipids measured from living *Loripes* and *Venerupis* are shown in Table 1. Most lipids from the thiotrophic *Loripes* were considerably more depleted (around 4.6 ‰) in ^{13}C than the respective lipids from *Venerupis.* Sterols, in contrast, were less depleted in *Loripes* compared to *Venerupis*. In both bivalves, gill tissue lipids were slightly more depleted (*c*. 2.8 ‰) than shell-lipids.

The carbon isotopic composition of subfossil lipids from the shell was not determined due to the limited number of sample.

For every sampling site, the $\delta^{13}C$ values of the bulk organic shell matrix from living specimens, empty shells and subfossil shells were more depleted in *Loripes* compared with *Venerupis* (Table 2). Depending on the sampling site, $\delta^{13}C$ values of *Loripes* ranged from -27.8 ‰ to -24.4 ‰ and in *Venerupis* from -24.1 ‰ to -17.5 ‰. Another heterotrophic bivalve *(Gastrana fragilis)* was also within the range of *Venerupis*.

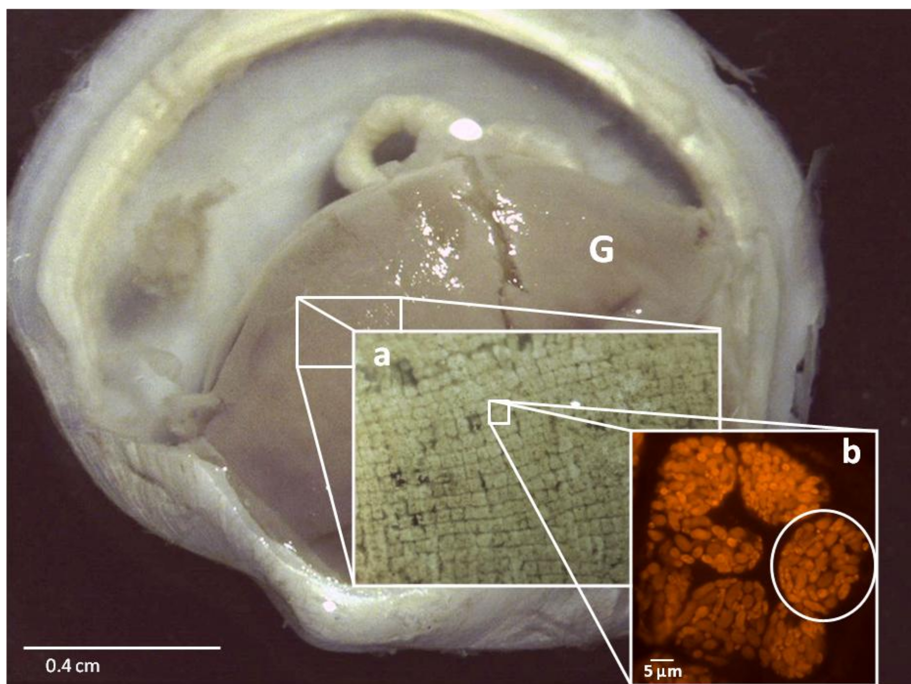

Fig. 1. *Loripes lacteus* gill tissue and symbiont localization. Large image: Gross anatomy of *Loripes lacteus* with respect to gill tissue (G) Inset: **a)** structure of gill tissue at low magnification, **b)** Fluorescence *in situ* hybridization image of bacterial symbionts (orange fluorescence), single bacteriocyte encircled.

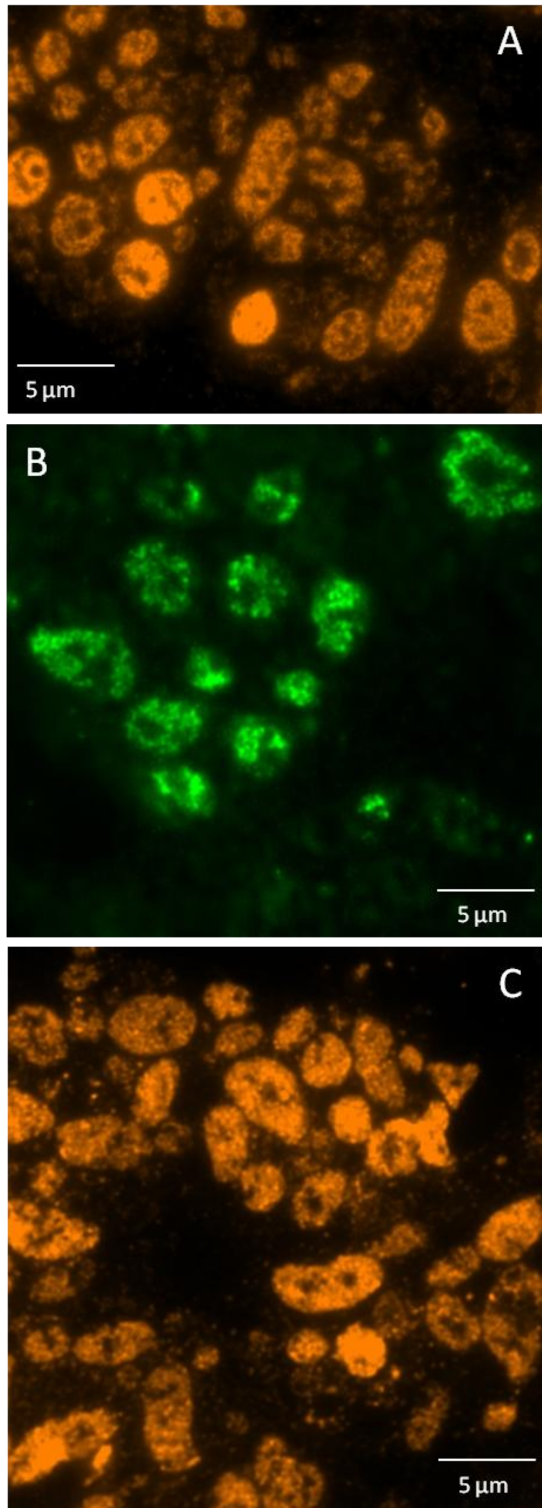

Fig. 2. Localization of three key enzymes in gill tissue of *Loripes lacteus* by immunofluorescence. Epifluorescence micrographs of 1 µm thin sections from gill tissue

A) RubisCO (AlexaFluor 546, anti-RbcLI antibodies), **B)** Glutamine synthetase GSI (FITC, anti-glutamine synthetase antibodies), **C)** APS reductase (AlexaFluor 546, anti-AprB antibodies).

Table 1. Total lipid $\delta^{13}C$ values (‰ relative to VPDB) for living bivalves from Sicily lagoon.

	Loripes lacteus		*Venerupis aurea*	
selected Lipids	$\delta^{13}C$ (‰) gill	$\delta^{13}C$ (‰) shell	$\delta^{13}C$ (‰) gill	$\delta^{13}C$ (‰) shell
Hexadecenoic acid	-32.4	-31.2	-27.7	-25.2
Hexadecanoic acid	-31.2	-29.2	-30.3	-27.5
Heptadecanoic acid	-34.0	n.d.	-27.2	n.d.
Octadecenoic acid	-34.3	-32.2	-30.3	-28.2
Octadecanoic acid	-34.7	-31.5	-29.0	-26.7
Dimethoxyoctadecane	-36.3	-31.0	-30.9	-27.2
Arachidonic acid	-35.2	n.d.	-28.9	n.d.
Eicosenoic acid	-34.7	-31.4	-29.1	-26.8
Docosapentaenoic acid	n.d.	n.d.	-27.9	-25.7
Docosadienoic acid	-36.3	-32.6	-28.1	-24.1
Cholest-5en-3β-ol (Cholesterol)	-25.9	-20.6.	-30.0	-24.3
24-Methylcholest-5,22-dien-3β-ol (Brassicasterol)	-26.3	n.d.	-30.1	n.d.
24-Methylcholest-5-en-3β-ol	-27.0	n.d.	-29.4	n.d.

Standard deviations of replicate measurements were generally < 0.5 ‰ and sometimes< 1 ‰. n.d., no data.

3.4.3 Nitrogen incorporation and related $\delta^{15}N$ values

The bacterial GS (GSI) was found to be expressed and located in the symbiotic bacteria (Fig. 2b) of *Loripes*. Western blot analysis with anti-GS (microbial, *E. coli*) antibodies confirmed that GSI was only expressed in gill tissue of *Loripes* and not in *Venerupis* extracts (Fig. 3). GS activities were measured in whole

soft tissue, as well as in foot and gill tissue of chemosynthetic *Loripes* and filter-feeding *Venerupis.* Kidney tissue of mouse served as positive control for GSII.
GS activity was detected in whole tissue extracts as well as in gill tissue of both species and in kidney extract, but not in foot tissue extracts (Fig. 4). Activity measured before heat inactivation at 60°C originates from both GSI and GSII, i.e. the values represent the sum of host and symbiont enzyme activities. After heating, 70-95 % of the activity still remained in gill and soft tissue of *Loripes,* whereas only residual activities ranging from 1-5 % were detected in the extracts of *Venerupis* (Fig. 4). The majority of activity after heating was attributed to the heat-stable GSI. After heating, kidney extract showed residual activity of 4 % (similar to the residual activity in *Venerupis*), whereas *E. coli* GSI had a remaining activity of 97 %. Thus, also in *Loripes* the majority of activity derived from the symbiotic GSI.
The influence of the expressed pathway of ammonium incorporation to the nitrogen isotopic composition of host tissue was verified by measuring $\delta^{15}N$ values: The $\delta^{15}N$ values of *Loripes* bulk organic matrix (from living specimens, empty shells and subfossils) ranged from 3 ‰ to 0.5 ‰ (Table 2), whereas values for *Venerupis* were less depleted, ranging from 10.1 ‰ to 4.2 ‰. Nitrogen and carbon isotope composition of chemotrophic vs. heterotrophic diets of shells from living bivalves, empty shells and subfossil shells were compared (Fig. 5). $\delta^{15}N$ plotted versus $\delta^{13}C$ show a consistent distinction between heterotrophic and thiotrophic isotopic values when at least two thiotrophic and heterotrophic samples from the same site were (identical colors in Fig. 5).

Table 2. $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ ratios of bulk organic matrix from the shells of living and subfossil *Loripes*, *Venerupis* (alive, empty shells, subfossil) and from living *Gastrana,* plus ratios from gill tissue.

	Sample	Isotopic composition $\delta^{13}C$ (‰) ±0.1‰	$\delta^{15}N$ (‰) ±0.2‰	$\delta^{34}S$ (‰) ±0.3‰	C/N-ratio
thiotrophic	*Loripes lacteus* (Sicily, alive)	-25.4	+3.0	-18.5	3.2
	Loripes lacteus (VeninceA, alive)	-24.4	+0.5	-17.6	3.1
	Loripes lacteus (subfossil)	-27.8	+0.7	-22.4	4.3
filter feeder	*Venerupis aurea.* (Sicily, alive)	-21.8	+10.1	+13.6	3.1
	Venerupis aurea. (VeniceA,empty shell)	-20.0	+5.3	-15.4	4.4
	Venerupis aurea (VeniceC, alive)	-17.7	+5.5	+7.6	3.1
	Venerupis aurea (VeniceC, empty shell)	-17.5	+5.5	+4.8	3.1
	Venerupis sp. (subfossil)	-24.1	+4.2	-30.8	4.1
	Gastrana fragilis (VeniceB, alive)	-18.2	+6.6	+4.9	3.0
	Loripes lacteus (Sicily, gill tissue)	-28.8	+1.4	-16.4	5.9
	Venerupis aurea (Sicily, gill tissue)	-21.7	+12.4	+18.0	3.9

Carbon isotopic compositions are reported relative to V-PDB. Nitrogen is reported relative to the atmospheric air standard and sulfur is reported relative to Canyon Diablo troilite standard

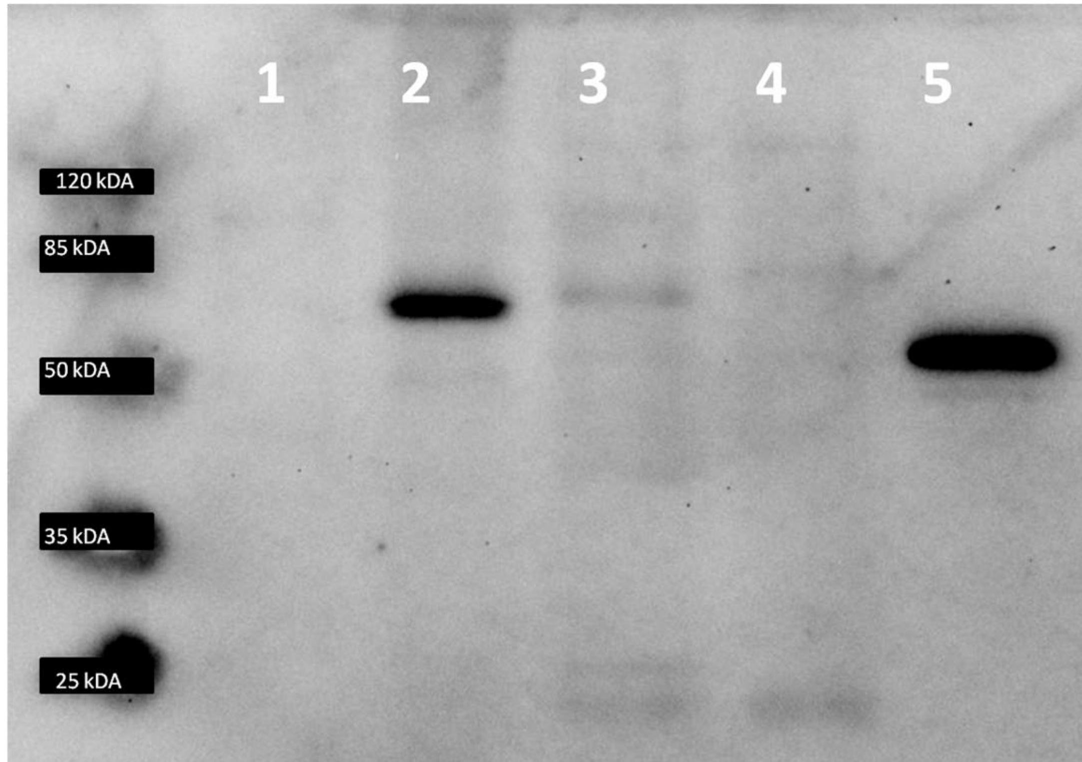

Fig. 3. Western blot analysis of protein extracts after SDS-PAGE: Anti-glutamine synthetase (microbial, *E. coli*) antibodies detect bacterial GSI, only in gill tissue extract of *Loripes lacteus.* Lane **1)** *Loripes* foot, **2)** *Loripes* gill, **3)** *Venerupis* foot, **4)** *Venerupis* gill, **5)** positive control (L-glutamine synthetase from *E. coli*).

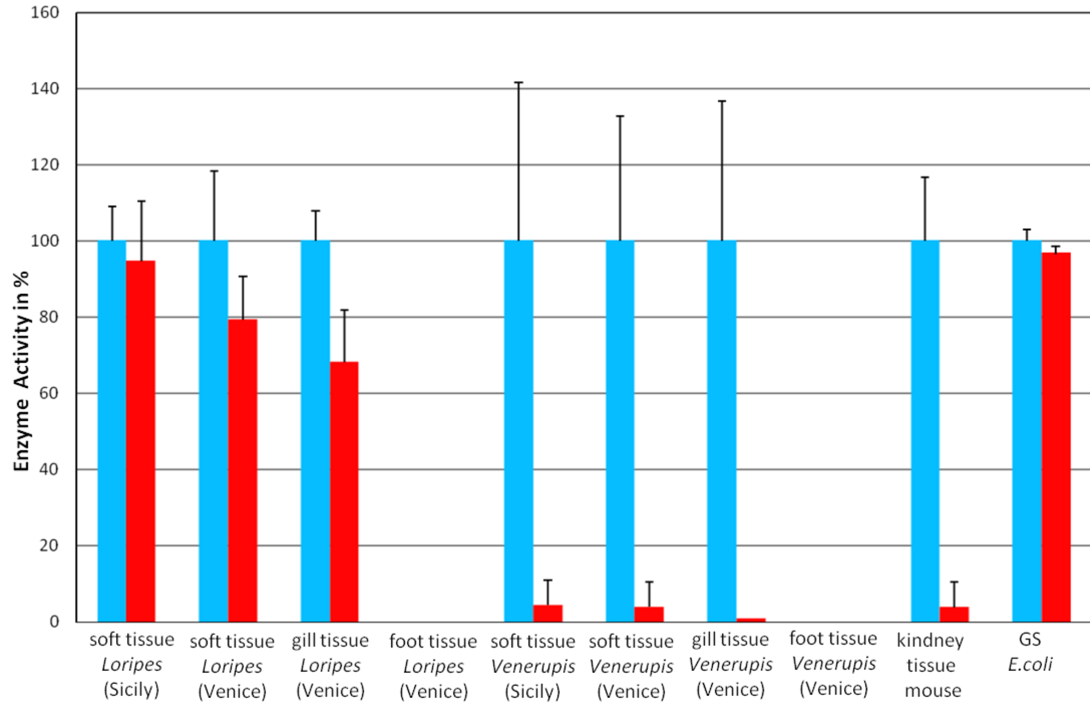

Fig. 4. Glutamine synthetase activity determined by the γ-glutamyltransferase assay in different tissue of bivalves. Activity was compared between untreated protein extracts (blue columns) and heat-treated protein extracts (red columns). Activity after heat treatment is given as a percentage of the value before treatment. The activity values are mean ± standard deviation of three independent experiments. (Sicily = Ganzirri lagoon; Venice = VeniceC: Sant'Andrea)

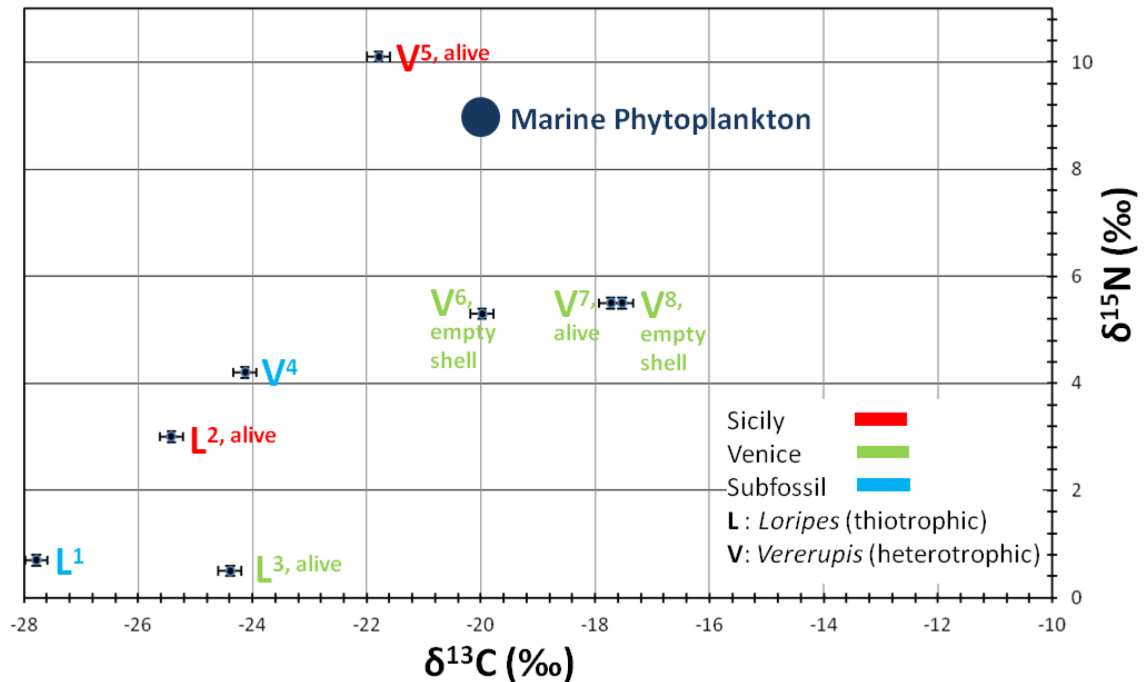

Fig. 5. Comparison of $\delta^{13}C$ and $\delta^{15}N$ in shell organic matrix from living bivalve shells, empty shells and subfossil shells. Different sampling sites are color coded: **red)** Sicily, **green)** Venice, **blue)** subfossil; *Loripes lacteus* **(L)**, *Venerupis aurea* **(V)**; **L1)** subfossil, **L2)** Sicily alive, **L3)** VeniceA alive, **V4)** subfossil, **V5)** Sicily alive, **V6)** VeniceA empty shell, **V7)** VeniceC alive, **V8)** VeniceC empty shell; (values for phytoplankton from Michener and Schell (1994). Measurement inaccuracy $\delta^{13}C$ +/-0.3; $\delta^{15}N$ +/-0.35.

3.4.4 Sulfur oxidation and related $\delta^{34}S$ values

Immunofluorescence revealed expression of the key enzyme APS reductase responsible for oxidation of sulfide within the gill symbionts (Fig. 2c). If ^{34}S-depleted inorganic sulfide is taken up by chemosymbionts via this pathway, a corresponding isotopic fractionation should be expected in the host biomass. The measured $\delta^{34}S$ values of bulk organic matrix (from living specimens, empty shells and subfossils) of sulfur-oxidizing *Loripes* and filter feeding *Venerupis* ranged from -30.8 ‰ to +18.4 ‰ (Table 2). Comparing the $\delta^{34}S$ values in bulk organic matrix and gill tissue, we observed differences of *c.* 2 to 5 ‰ (*Loripes* gill -16.4 ‰, *Loripes* shell -18.5 ‰; *Venerupis* gill +18.0 ‰; *Venerupis* shell +13.6 ‰). Values were more depleted in living *Loripes* than in living *Venerupis*, whereas this pattern was not found in empty and subfossil shells (Fig. 6).

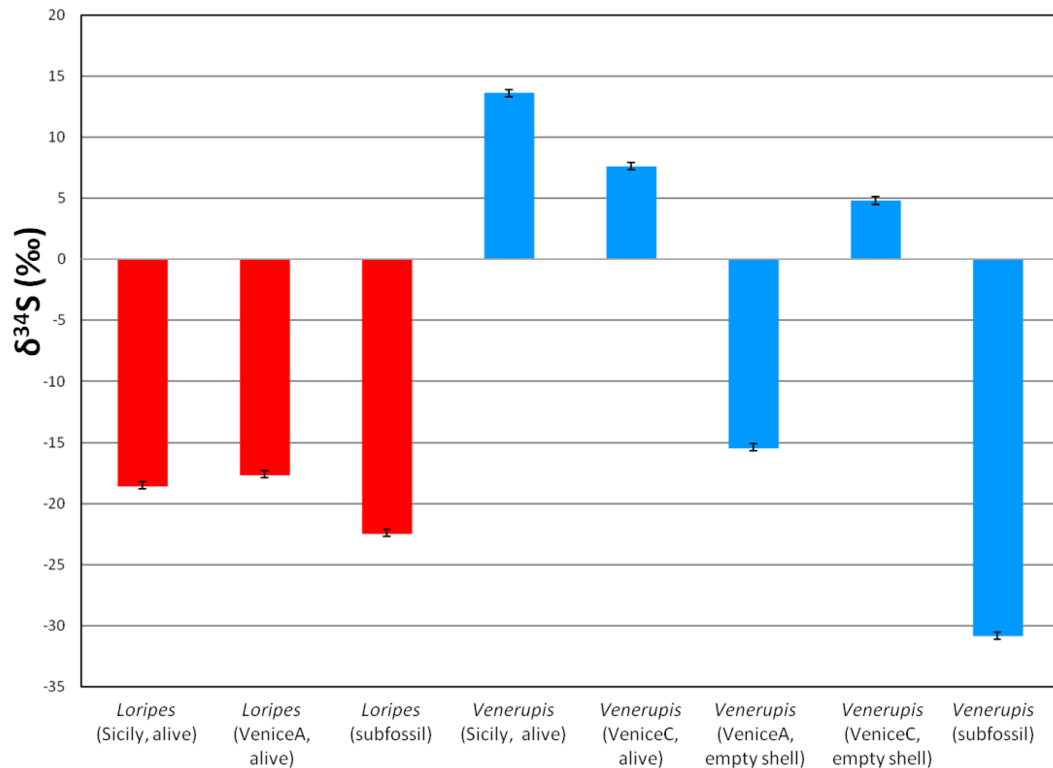

Fig. 6. $\delta^{34}S$ of organic sulfur in bulk organic matrix from shells of living bivalves, empty shells and subfossil shells. Species are color coded: **red)** *Loripes lacteus*, **blue)** *Venerupis aurea*.

3.5 Discussion

3.5.1 Life styles of the bivalves Loripes lacteus and Venerupis aurea

16S rRNA gene sequence analysis and FISH (Fig. 1b) confirmed the high abundance of the *L. lacteus* gill symbiont in gill tissue, which is a prerequisite for all further studies. The detected *Synechococcus*-related sequence represents one predominant cyanobacterial genus, which is common in the marine environment. *Synechococcus* was not observed as endosymbionts in this study. It may therefore be expected that the cyanobacterium adhere at the prepared gill tissue and was amplified as a 'contamination' from the environment. In contrast, it was confirmed that *V. aurea* as filter feeder did not contain any endosymbiotic bacteria.

3.5.2 Sulfur isotopes as biosignatures of thiotrophic metabolism?

We were able to localize three expressed key enzymes for carbon and nitrogen assimilation as well as for sulfur metabolism within the *L. lacteus* gill symbiont. The detection of APS reductase in the cytoplasm of the symbionts (Fig. 2c) shows that the organism use the reverse-operating enzyme of the sulfate-reduction process to generate energy from sulfide oxidation, which is in accordance with results from Herry et al. (1989) and Duperron & Fiala-Médioni (2007), who detected APS reductase gene sequences in *Lucinoma kazani* and measured activity of APS reductase in gill tissue of *Loripes*.

Hence the diet of *Loripes* is based on the thiotrophic metabolism of its endosymbionts. The sulfur from free sulfide or possibly insoluble iron sulfide (Dando et al., 1994), taken up by the symbionts from the sediment, is depleted in ^{34}S as a result of microbial sulfate reduction in the anoxic sediment (Kaplan et al., 1963; Fry et al., 1988; Ohmoto et al., 1990; Vetter & Fry, 1998; Aharon & Fu, 2000; Joy et al., 2004). The $\delta^{34}S$ values of bulk organic matrix from the shells of living *Loripes* was around -18 ‰ compared with $\delta^{34}S$ values of living *Venerupis* and *Gastrana* ranging from 4.9 ‰ to 13.6 ‰ (Table 2), the latter indicating another source than sulfate reduction-derived sulfides for organic sulfur in the biomass. In *Loripes,* the sulfide-based depleted sulfur is possibly directly assimilated by the sulfide-oxidizing symbionts, via o-acetylserine in cysteine. In general, cysteine serves as a central metabolite for the incorporation in different cell components, mainly in proteins. In addition, the generated APS could serve as a central pool for the uptake and delivery of depleted sulfur in cell material of the symbionts. Because the diet of *Venerupis* and *Gastrana* is based only on filter feeding, no depleted sulfur is found in organic macromolecules of these mollusks. The sulfur compounds of their diet phytoplankton derive from sea-water sulfates, which differ markedly from the $\delta^{34}S$ ratios of sulfides in sediments (Kaplan et al., 1963; Trust & Fry, 1992; Michener & Schell, 1994).

Regardless, the strong difference up to 30 ‰ between thiotrophic and heterotrophic bivalves (Table 2) indicates whether their diets were based on thiotrophic endosymbionts or on filter feeding. However, this difference could only be confirmed for shells from living specimens, as recent empty shells and

subfossil specimens from any species were found to have strongly depleted $\delta^{34}S$ values (Fig. 6). The decrease of $\delta^{34}S$ values after the death of the organisms (Fig. 6) is probably due to the instability of the sulfur-containing amino acids, namely methionine and cysteine (Jones & Vallentyne, 1960). Moreover, sulfides derived from proteolysis and from bacterial sulfate-reduction during soft tissue degradation may lead to further depletion in ^{34}S. Hence, although the differentiation between thiotrophic and filter-feeding lifestyle is excellently reflected by sulfur isotopes in living bivalves, the $\delta^{34}S$ ratio is not a reliable signature for this discrimination in dead or even subfossil specimens (Fig. 6). Previous studies have shown only $\delta^{34}S$ data of the shell protein matrix of modern bivalves, where sulfur isotopes may be useful biosignatures, although for subfossil and fossil bivalves no $\delta^{34}S$ data were described (O´Donnell et al., 2003; Mae et al., 2007).

3.5.3 Carbon isotopes as biosignatures of chemoautotrophically fixed carbon

In addition to APS reductase also RubisCO was detected in the cytoplasm of the endosymbionts (Fig. 2a). Thus, the autotrophic fixation of CO_2 via the Calvin-Benson cycle could be confirmed at the level of enzyme expression. This is also in agreement with the specific $\delta^{13}C$ ratios in lipids and the bulk organic matrix of the shell, as *Loripes* obtains organic carbon from its symbionts (Herry & Le Pennec, 1989). The bulk organic matrix from shells is mainly composed of proteins, which are generally enriched relative to dietary carbon by 0.7 ‰ to 1.4 ‰, whereas lipids were found to be depleted relative to their diet by 3 ‰ (DeNiro & Epstein 1977; Crenshaw, 1980).

The $\delta^{13}C$ of the bulk organic matrix from *Loripes* shells exhibits values ranging from -24.4 ‰ to -27.8 ‰ (Table 2). Thus, the organic shell matrix is slightly less depleted in ^{13}C than in other chemoautotrophic bivalves, which range from -27 ‰ to -35 ‰ (Childress & Fisher 1992; Robinson & Cavanaugh 1995; Cavanaugh & Robinson 1996). Mae et al. (2007) reported a difference in $\delta^{13}C$ of 3 ‰ between organic matrix and soft tissue in the seep-related bivalves *Calyptogena* and *Bathymodiolus*. In our study, a difference of 3.4 ‰ was calculated between gill tissue and organic shell matrix, suggesting the non seep-related *L. lacteus* to be similar to other chemosymbiotic bivalves with

respect to thiotrophy and carbon flow from symbionts to host (Fisher, 1990). As a filter-feeding bivalve, *Venerupis* obtains carbon mainly from phytoplankton. The $\delta^{13}C$ ratios measured in organic matrix from *Venerupis* (-24.1 ‰ to -17.5 ‰) are higher than in *Loripes* as RubisCO from photosynthetic organisms like phytoplankton leads to less depleted carbon than the RubisCO from chemoautotrophs (Ruby et al., 1987). The $\delta^{13}C$ values of subfossil and living *Venerupis* (between -24.1 ‰ and -17.5 ‰) was in the range of other bivalves living in marine environments (Mae et al., 2007 and references therein).

The same $\delta^{13}C$ patterns were found for total lipids. Generally, the lipid isotope ratio is more depleted in *Loripes* than in *Venerupis* with exception of sterols (Table 1). An equal isotopic composition of sterols in heterotrophic and symbiotic bivalves was also described by CoBabe & Pratt (1995). Lucinids are functionally capable of filter-feeding (Duplessis et al., 2004); the high $\delta^{13}C$ values of sterols from *Loripes* lipids imply that these compounds in *Loripes* and in the filter feeder *Venerupis aurea* derive from eukaryotic phyto- and zooplankton (Donval et al., 1988; Conway et al., 1991; CoBabe et al., 1995). This is also supported by the fact that sterol distribution was similar in both bivalves.

The striking difference between the $\delta^{13}C$ values in both the organic matrix and lipids of the two bivalves show that the carbon isotopic composition is based on the major food source. Remarkably, the difference of $\delta^{13}C$ values is also stable in the organic matrix over geological time scale, since the subfossil samples of *Loripes* and *Venerupis* possess a similar difference around 4 ‰ between $\delta^{13}C$ values of living bivalves.

3.5.4 Assimilation of ammonium and related $\delta^{15}N$ values as biosignatures for endosymbiosis

Shell matrix $\delta^{15}N$ values were distinct between the subsets of thiotrophic and filter-feeding bivalves. However, these values also varied between different sampling sites. Thus the absolute values seem to be insufficient as a signature (cf. Versteegh et al., 2011). However, when data from *Loripes* and *Venerupis* samples taken from the same site were compared, the difference in ^{15}N depletion, ranging between 3.5 ‰ and 7 ‰, was evident.

For some symbioses the assimilation of either nitrate (e.g in *Riftia*) or ammonium (e.g. in *Solemya*) as inorganic nitrogen sources has been described (Lee & Childress, 1994). The method of ammonium assimilation is not well understood, but in *Loripes* used in this study, it can be assumed that it is mainly performed by the bacterial symbionts. Western blotting shows the expression of the key enzyme for ammonium assimilation (GSI) and additional immunofluorescence microscopy reveals localization of GSI in the cytosol of the endosymbionts (Fig. 2b and Fig. 3). Enzyme activity measurements allowed us to distinguishing between the activity of the host (GSII) and symbiont (GSI) enzyme. Activities measured before heating the extracts reflect host and/or symbionts GS, whereas after heating the prokaryotic GSI enzyme is still active (Merrick & Edwards, 1995). As GSI activity was the dominant GS activity expressed in *Loripes* (Fig. 4), it could be assumed that conversion of ammonium to organic nitrogen takes place within the symbionts. In contrast to our findings, in *Solemya velum*, another thiotrophic bivalve, no GSI activity was detected (Lee et al., 1999). As discussed by the authors, in some symbioses, e.g. between nitrogen-fixing Rhizobia and plants, the expression of GSI in the symbionts is repressed and the host assimilates ammonium by its own. If ammonium assimilation is performed primarily either by the hosts or by the symbionts, the presence and activities of GSI vs. GSII may reflect whether symbionts were either digested by the host or if organic compounds are transferred from living symbionts to the host (Lee et al., 1999). If the host GSII assimilates ammonium, and GS activity is repressed in symbionts, organic carbon will be mainly stored as carbohydrates, whereas biosynthesis of amino acids is reduced. Thus the host could "milk" the surplus carbohydrates from the symbionts. Otherwise, the expression of GSI and the assimilation of ammonium by the symbionts may be an indicator that transfer of organic compounds to the host occurs by digestion of symbionts, which appears to be the case here for *Loripes*. Herry et al. (1989) support such a scenario of direct digestion by bacterial lysis.

It is questionable if ammonium assimilation by either GSI or GSII leads to different depletion of ^{15}N. In both investigated bivalves species, GS was active in the gill tissue. But the role of GSII activity in the gill of *Venerupis* may be

more related to detoxification of ammonia than to glutamine biosynthesis (Meistertzheim et al., 2007). Thus, the main nitrogen source in *Venerupis* appears to derive from the planktonic protein, not from ammonium assimilated via GSII. It is known that the $\delta^{15}N$ ratio increases about 3.4 ‰ per trophic level (Minagawa & Wada, 1984). The higher trophic level of *Venerupis* compared with *Loripes* is probably the reason for the higher $\delta^{15}N$ values measured here: the uptake and assimilation of ammonium by bacteria generally results ^{15}N depletion. This depletion is not just an effect of the reaction catalyzed by GSI or glutamate dehydrogenase, but is also caused by active transport of ammonium or passive diffusion of ammonia across the bacterial membrane (Hoch et al., 1992). Thus, the the depleted $\delta^{15}N$ values of *L. lacteus* (near the base of the food chain) are explained by diet, which is based on bacterial biomass of the endosymbionts.

3.5.5 $\delta^{13}C$ and $\delta^{15}N$ isotopic data as signatures for chemosymbiosis

During organic matter degradation C/N ratios generally increase with increasing maturity. Bulk organic shell matrix with C/N ratios between 2.9 to 3.2 measured in this study represents 'fresh' (no diagenetic alteration, Ambrose, 1994) sampling material, supporting the applicability of $\delta^{34}S$ ratios. Older postmortem (higher alteration) shells and subfossil samples display C/N values higher than 4.1 coupled to $\delta^{34}S$ values much more depleted than in living organisms (Fig. 6, Table 2). Depending on the postmortem chemical and biological conditions in the depositional environment, the diagenetic alterations differ. Thus $\delta^{34}S$ values are only a useful parameter for thiotrophic symbiosis in shells from living bivalves. Our work clearly shows that the combination of carbon and nitrogen isotope data from bulk organic shell matrix of different bivalve species taken from the same sampling site are required to discriminate whether their diet is based on chemosynthesis or filter-feeding. It is also possible to detect ancient chemosymbiosis in subfossil specimens based on these data. Mae et al. (2007) reported highly depleted $\delta^{13}C$ values of -28.5 ‰ and $\delta^{15}N$ of -1.4 ‰ for fossil chemosynthetic Vesicomyidea, which is within the range of data determined here, with $\delta^{13}C$ of -27.8 ‰ and $\delta^{15}N$ of 0.7 ‰ for subfossil *Loripes.* For fossil chemosynthetic Mytilidae, in contrast, Mae et al. (2007) described $\delta^{13}C$ of -

23.1 ‰ and $\delta^{15}N$ of +4.2 ‰ and speculated about a chemotrophic origin. These values, however, were more within the range of our data for heterotrophic *Venerupis* with $\delta^{13}C$ of -24.1 ‰ and $\delta^{15}N$ of +4.2 ‰, indicating that shell matrix $\delta^{13}C$ values in this range can arise with no chemosynthetic involvement.
Together, our data show that the $\delta^{13}C$ and $\delta^{15}N$ values of a single species alone are not reliable to identify ancient chemosymbiosis. The food source and the environmental conditions of filter-feeding and chemotropic bivalves is related to local productivity, hydrodynamic as well as temperature conditions, hence the isotopic carbon and nitrogen composition varies from site to site (cf. Dattagupta et al., 2004). To overcome this drawback, at least two different species, one definitely representing a filter-feeding lifestyle, must be sampled from the same site. Only then can stable isotopes be used as indicators of ancient chemosymbiosis or other diets.

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4. The isotopic biosignatures of photo- *vs.* thiotrophic bivalves: are they preserved in fossil shells?

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4.1 Abstract

Bivalves, that harbor thiotrophic or phototrophic symbionts, exhibit characteristic isotopic signatures in their bulk organic shell matrices. Phototrophic (*Fragum fragum, Fragum unedo*, *Tridacna maxima*), thiotrophic (*Codakia tigerina, Fimbria fimbriata, Anodontia sp.*) and non-symbiotic (*Tapes dorsatus, Vasticardium vertebratum, Scutarcopagia sp.*) marine bivalves were used as model organisms in this study. They were collected from shallow waters off North Stradbroke Island and One Tree Island situated at the east coast of Queensland, Australia. To establish biosignatures for the detection of distinctive symbioses in ancient bivalves, the isotopic composition of lipids ($\delta^{13}C$) and bulk organic shell matrix ($\delta^{13}C$, $\delta^{34}S$, $\delta^{15}N$) from shells of thio-, photo- or non-symbiotic bivalves was obtained for comparative analysis. Compared to living bivalve shells, the composition and $\delta^{13}C$-value differences of empty shells indicate that the majority of shell-lipids from empty shells derived from a decomposing prokaryotic community. The use of lipids from ancient shells for the reconstruction of the bivalve's life style appears therefore to be restricted.

$\Delta^{13}C$ values of bulk organic shell matrices, most likely representing mainly original shell protein/chitin biomass, were depleted in thio- and phototrophic bivalves compared to non-symbiotic bivalves. However, just ^{13}C-depletion alone was not

sufficient to discriminate between thio- or photo-trophic lifestyles of the bivalve species studied. The bulk organic shell matrix showed the highest depletion of $\delta^{15}N$ down to -2.2 ‰ for thiotrophic bivalves. Thus, combined $\delta^{13}C$ and $\delta^{15}N$ values are useful signatures to differentiate between thio-, phototrophic and non-symbiotic lifestyle of bivalves. However, the use of these signatures for ancient bivalves is limited by the conservation of the bulk organic shell matrix in fossils. The responsible alteration process was clearly shown by detailed microscopic analyses of a fossil (late Pleistocene) *Tridacna maxima* shell, demonstrating a severe loss of quantity and quality of bulk organic shell matrix with time.

4.2 Introduction

Different prokaryotes are frequently found as symbionts in marine bivalves of various families, e.g. Solemyidae, Mytilidae, Thyasiridae, Lucinidae, Teredinidae (Distel et al., 2002; Dubilier et al., 2008; Taylor & Glover 2010; Oliver et al., 2013). Some marine bivalves also live in symbiosis with eukaryotic algae. These “photosymbiotic” bivalves harbor dinoflagellates of the genus *Symbiodinium* (Blank & Trench, 1986), which are commonly located intracellularly in a special tubular system connected to the stomach situated in the siphonal mantle or/and in gill tissue and also sometimes in foot tissue (Blank, 1986; Yonge, 1936; Purchon, 1955; Stasek, 1961; Kawaguti, 1950; 1968; 1983; Hartman & Pratt, 1976; Jacobs & Jones, 1989; Jones & Jacobs, 1992; Norton et al., 1992; Ohno et al., 1995; Persselin, 1998, Vermeij, 2013). Until now photosymbiosis in modern bivalves is known from the taxa of Cardiidae (Tridacninae, Fraginae and Clinocaradiinae) and from Trapeziidae (*Fluviolanatus*) as well as from the freshwater Unionidae (*Anodonta* and *Unio*, with the zoochlorellae as symbionts, Pardy, 1980). Besides the freshwater bivalve *Anodonta* and *Unio* (Vermeij, 2013) marine photosymbiotic bivalves live in the Indo-West Pacific region (Kirkendale, 2009). In contrast to the chemoautotrophic or methanotrophic symbionts using H_2, H_2S or CH_4 as electron donors, the symbiotic algae use the energy of sunlight to fix CO_2 or HCO_3^- into organic carbon. The host benefits from the products of symbiont photosynthesis (e.g. glucose, glycerol in giant clams). In exchange algae symbionts gain protection and some metabolites from their host (Yonge, 1975; 1981; Janssen, 1992; Berry & Playford, 1998; Ishikura et al., 1999). The architecture of

bivalve shell is often adapted to photosymbiotic lifestyle. Translucent windows, for example, are an adaption of the shell microstructure, which allow the light to reach symbiont-bearing tissue (Watson & Signor, 1986). For a detailed review on bivalves morphological adaptations see Vermeij (2013).

Studies of modern photosymbiotic bivalves have led to speculations about putative photosymbiotic lifestyles of ancient bivalves (Kauffman, 1969; Philip, 1972; Cowen, 1983; Jones et al., 1988; Seilacher, 1990; MacLeod & Hoppe, 1992; Savazzi, 2001). Tridacninae occur since the Late Eocene and Fraginae since the Late Miocene (Keen, 1980; Romanek et al., 1987; Vermeij, 2013). Whether or not the ancient relatives of modern photosymbiotic Tridacninae and Fraginae also possessed a symbiotic relationship to algae is still a matter of discussion. An ancient symbiotic lifestyle of these bivalves was deduced on the basis of palaeoecology and structural features of their shell (e.g. Ohno et al., 1995; Schneider, 1998a). Jones et al. (1986) analyzed stable oxygen and carbon isotopes of shell carbonate from *Tridacna maxima* and showed that the symbiotic relationship with algae in mollusks lead to a higher depletion of skeletal $\delta^{13}C$ as compared to non-symbiotic mollusks. No difference was observed between $\delta^{18}O$ values of photosymbiotic vs. filter-feeding (non-symbiotic) mollusks. A later study of Romanek and Grossman (1989) reported that the presence of symbionts in *T. maxima* has no identifiable effect on the isotopic composition of shell carbonate compared to other bivalves. Besides analyses of $\delta^{18}O$ and $\delta^{13}C$ of shell carbonate, the isotopic composition of organic compounds within the shell allows to demonstrate chemosymbiosis in modern and fossil bivalves. One approach is the measurement of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ in whole organics within the shell (O'Donnell et al., 2003; Mae et al., 2007; Dreier et al., 2012). In addition, some studies analyzed individual shell-lipids of chemotrophic bivalves and showed that these compounds were more depleted in ^{13}C as compared to those of non-symbiotic bivalves (CoBabe & Pratt 1995; Conway & Capuzzo, 1991; Dreier et al., 2012). To identify specific signatures in modern and fossil bivalve shells that in turn determines the animal's lifestyle, we analyzed the isotopic compositions of (i) the whole (bulk) organic shell matrix and (ii) of individual shell-lipids, and compared these data between non-symbiotic, chemotrophic (esp. thiotrophic) and phototrophic bivalves. Furthermore, for a critical assessment of diagenetic shifts in shell isotopy, lipid content and bulk organic shell matrix quality, shells from living bivalves, empty-sampled shells, and a fossil shell were analyzed isotopically and microscopically.

4.3 Materials and methods

4.3.1Sample collection

Empty shells and living specimens of bivalves were collected by hand from a depth of 2-3 m from two locations at the east coast of Australia during November 2012. *Fragum unedo* (phototrophic*), Anodontia sp.* (thiotrophic)*, Vasticardium vertebratum* (non-symbiotic) and *Tapes dorsatus* (non-symbiotic) were obtained from the seagrass beds off Dunwich at North Stradbroke Island, Moreton Bay, Australia (S27°29'42'' E153°23'60''). *Fragum fragum* (phototrophic), *Codiaka tigerina* (thiotrophic), *Fimbria fimbriata* (thiotrophic) and *Scutarcopagia sp.* (non-symbiotic) were sampled at the sandy intertidal zone off One Tree Island, The Great Barrier Reef, Australia (S23°30'23'' E152°5'28''). Live-sampled specimens were kept frozen at -15 °C until dissection in the lab.

Empty (modern, non-fossil) *Tridacna maxima* shell (valve ca. 12 cm long) from One Tree Island was provided by Robert Moore under permit number: G99-192 (GBRMPA). A fossil shell (original shell mineral not recrystallized) of *Tridacna maxima* (GZG.INV.76418) from Pleistocene, north of Dahab, Sinai, Egypt, was provided by Mike Reich (Geoscience Centre, Museum, Collections & Geopark, University of Göttingen).

All samples and their abbreviations are listed in Table 1.

Table 1. Sample collection used for this study with corresponding abbreviations.

species	sampling site	modern shell sampled alive **(a)**	modern shell sampled empty **(e)**	fossil shell	abbreviation
Fragum unedo (phototrophic)	North Stradbroke Island	**X**	**X**		FU*(a or e)
Anodontia sp. (thiotrophic)	North Stradbroke Island		**X**		AN*(e)
Vasticardium vertebratum (non-symbiotic)	North Stradbroke Island		**X**		VV*(e)
Tapes dorsatus (non-symbiotic)	North Stradbroke Island	**X**	**X**		TD*(a or e)
Fragum fragum (phototrophic)	One Tree Island		**X**		FF# (e)
Codakia tigerina (thiotrophic)	One Tree Island		**X**		CT#(e)
Fimbria fimbriata (thiotrophic)	One Tree Island		**X**		FI#(e)
Scutarcopagia sp. (non-symbiotic)	One Tree Island		**X**		SC#(e)
Tridacna maxima (phototrophic)	One Tree Island		**X**		TM#(e)
	Sinai, Egypt			**X**	

4.3.2 16S rRNA and ITS gene sequence analysis

Genomic DNA was extracted from ca. 100 - 450 mg of gill or mantle tissue from one specimen of *F. unedo, F. fragum, Anodontia sp.* and *F. fimbriata,* respectively, using the NucleoSpin® Soil kit (Macherey-Nagel, Düren, Germany). The 16S rRNA genes were PCR -amplified as described by Wrede et al. (2011) using 8f (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492r (5′-GTTACCTTGTTACGACTT-3′) (Alain et al., 2006) as forward and reverse primers, respectively. The ITS2 of nuclear ribosomal RNA genes from *Symbiodinium spp.* was amplified as described by LaJeunesse & Trench (2000) using ITSintfor2 (5`-GAATTGCAGAACTCCGTG-3‘) and ITS2 (5‘-GGGATCCATATGCTTAAGTTCAGCGGGT-3’) as forward and reverse primers respectively. Amplification products were sequenced by the Göttingen Genomics Laboratory (University of Göttingen, Germany).

4.3.3 Isotopic analysis of bulk organic shell matrix

Recent shells (empty shells and shells from bivalves sampled alive) taken from two to eight specimens (16 for *Anodontia sp*. because of the small size) and one shell from fossil *T. maxima* (Pleistocene) were cleaned and homogenized as described for extraction of lipids (see above). The shell powder (around 8-12 g) was placed into dialysis tubings (Serva, Heidelberg, Germany) and then dialyzed (Mae et al., 2007) against sterile 0.5 M EDTA (pH 7.4; Carl Roth GmbH, Karlsruhe, Germany) at room temperature. EDTA solution was changed every 2 days. After 2 - 3 weeks, the mineral phase was dissolved and the remaining bulk organic shell matrix appeared as beige flakes. Then the samples were exhaustively dialyzed against sterile double-distilled water. Finally, the bulk organic shell matrix was freeze-dried. The dry bulk organic shell matrix was analyzed for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ using Vario EL III GVI Isoprime isotope-ratio MS (Sieper et al., 2006) at the Laboratorium für Stabil-Isotopenanalytik (Schweitenkirchen, Germany). The analytical errors were $^{13}C \pm 0.1$ ‰, $^{15}N \pm 0.2$ ‰, $^{34}S \pm 0.3$ ‰ respectively.

4.3.4 Lipid extraction and isotopic analysis

Empty shells from One Tree Island: *C. tigerina* (n= 3)*, F. fimbriata* (n= 2)*, F. fragum* (n= 5)*, T. maxima* (n= 1) and *Scutarcopagia sp*. (n= 2) were cleaned under binocular microscopy, using needle, scalpel, brass brush and sterile double-distilled water to

remove organic and mineral surface encrustation from the shell surface. After cleaning, shells were washed twice in sterile double-distilled water, pulverized and homogenized using a porcelain mortar and pestle. Homogenates were subjected to alkaline hydrolysis with 6% KOH in methanol for 2 h at 70 °C by successive sonication, followed by extraction of neutral lipids with *n*-hexane (three times). The remaining alkaline solution was acidified to pH 1 – 2 with HCl and extracted three more times with *n*-hexane. The combined extracts were evaporated to near dryness and reacted with a trimethylchlorosilane/MeOH mixture for 90 min at 80 °C to convert fatty acids to their methyl esters derivatives. The neutral lipid were silylated with N,O-bis(trimethylsilyl)trifluoroacetamide for 90 min at 80 °C and dissolved in *n*-hexane with activated copper chips (to remove elemental sulfur). Compounds were identified via GC-MS analyses (Varian CP-3800 chromatograph coupled to a 1200L mass spectrometer; Agilent, Santa Clara, CA). $\delta^{13}C$ values of selected lipids (Table 2) were measured using a Trace GC gas chromatograph coupled to a Delta Plus isotope-ratio mass spectrometer (both Thermo Scientific, Waltham, MA). The combustion reactor contained CuO, Ni and Pt and was operated at 940 °C. The stable carbon isotope compositions are reported in the delta notation ($\delta^{13}C$) vs. the V-PDB standard.

4.3.5 Light and electron microscopy

For scanning electron microscopy (SEM) modern (empty shell) and fossil pieces of *T. maxima* shell (around 0.5 cm in diameter) were fixed at 4 °C with 2.5 % glutaraldehyde in 0.5 M EDTA pH 7.4 (to start decalcification) for ca. 48 h. After fixation, pieces were dehydrated in an ascending series of ethanol concentrations, mounted on SEM sample holders and sputtered with platinum-palladium (13.0 nm). Scanning electron micrographs were taken with a SEM LEO 1530 Gemini (Zeiss, Oberkochen, Germany).

In addition, a piece of fossil and modern (empty) *T. maxima* shell was completely decalcified with in 0.5 M EDTA pH 7.4 and one part of remaining bulk organic shell matrix was used for light microscopy. Samples were stained with Calcofluor white. Epifluorescence microscopy was performed with a Zeiss Axioskop 40 equipped with an Axiocam MRm camera (Carl Zeiss, Göttingen, Germany) by using filter set 43 and 49 (Carl Zeiss). The other part was fixed and processed as described for SEM.

Pieces (ca. 1 cm in diameter) of the dehydrated *T. maxima* shell were embedded in LR White Resin medium grade (London Resin Company Ltd, Berkshire, England). Thin sections (ca. 300 µm thickness) were made and attached to a glass slides with araldite. Sections were decalcified in sterile 0.5 M EDTA (pH 7.4; Carl Roth GmbH, Karlsruhe, Germany) at room temperature until transparent parts are visible. Fluorescence images were taken with a Zeiss Axioskop 40 microscope and filter set 43 (Zeiss, Göttingen, Germany).

4.4 Results

4.4.1 Identification of symbionts by 16S rRNA and ITS gene sequence analysis

The presence of expected microbial symbionts in living *Anodontia sp., F. fimbriata, F. fragum and F. unedo* was verified by analysis of the amplified 16S rRNA and ITS gene sequences from gill and mantle tissue. The retrieved 16S rRNA gene sequence from gill tissue of *Anodontia sp* displayed 99 % similarity to a sequence from the bacterial endosymbiont (Piscirickettsiaceae, accession no. EU983577.1) of *Anodontia ovum.* The 16S rRNA sequence from gill tissue of *F. fimbriata* displayed 97 % similarity to a sequence from an uncultured bacterium clone V1SC07b71 from a hydrothermal vent microbial mat (accession no. HQ153956.1). The ITS2 sequence from mantle tissue of *F. fragum* displayed 99 % similarity to a sequence from *Symbiodinium sp.* clade C (accession no. KC631400.1) and sequence from *F. unedo* displayed 100 % similarity to a sequence from *Symbiodinium sp.* clade C (accession no. KC631409.1).

4.4.2 Isotopic composition of shell organics related to thiotrophic- vs. photosymbiotic lifestyle of bivalves

With the aim to identify potential isotopic differences between thio-, photo and non-symbiotic bivalves, the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values of bulk organic shell matrices and the compositions and $\delta^{13}C$ values of selected shell-lipids were determined.

Bulk analyses

The bulk organic shell matrices of phototrophic and thiotrophic bivalves is generally more depleted in ^{13}C than in non-symbiotic bivalves (Fig. 1, Table 3), ranging from -31.0 ‰ to -25.5 ‰. An exception was the $\delta^{13}C$ value of the phototrophic *T. maxima*

bulk organic shell matrix which was within the range of analyzed non-symbiotic bivalves (-20.1 ‰ to -22.0 ‰).

$\delta^{15}N$ values of all thiotrophic bivalves were lower (0.1 ‰ to -2.2 ‰) than those of photo-and non-symbiotic bivalves bulk organic shell matrix (Table 3). When carbon and nitrogen isotopic compositions of non-symbiotic, thio-and phototrophic bivalves were compared by plotting $\delta^{15}N$ versus $\delta^{13}C$ values (Fig. 1) three groups became evident, namely high $\delta^{13}C$/high $\delta^{15}N$ (non-symbiotic bivalves and young phototrophic *T. maxima*), low $\delta^{13}C$/high $\delta^{15}N$ (phototrophic *F. fragum*), and low $\delta^{13}C$/low $\delta^{15}N$ (thiotrophic Lucinidae).

$\Delta^{34}S$ values in the bulk organic shell matrix from thiotrophic bivalves were much lower than in other bivalves (Table 3, Fig. 2).

The remaining amount of bulk organic shell matrix obtained from dialysis of the fossil bivalve (*T. maxima*) was too small for analysis of the isotopic composition.

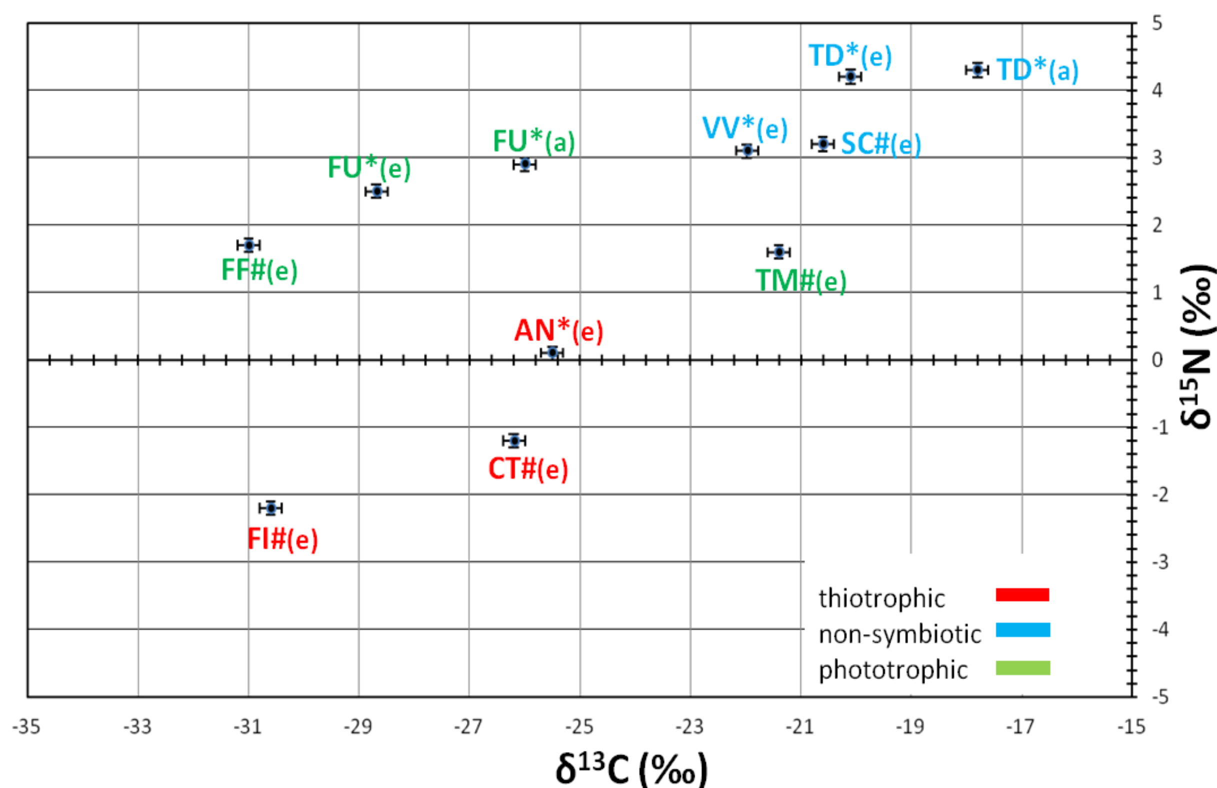

Fig. 1. Comparison of $\delta^{13}C$ and $\delta^{15}N$ from bulk organic shell matrix of living bivalve shells (a) and empty shells (e). Different lifestyles are color coded: (red) thiotrophic, (green) phototrophic, (blue) non-symbiotic. Abbreviations according to Table 1. (*= North Stradbroke Island, #= One Tree Island). Measurement inaccuracy: $\delta^{13}C$ ±0.1 ‰; $\delta^{15}N$ ±0.2 ‰.

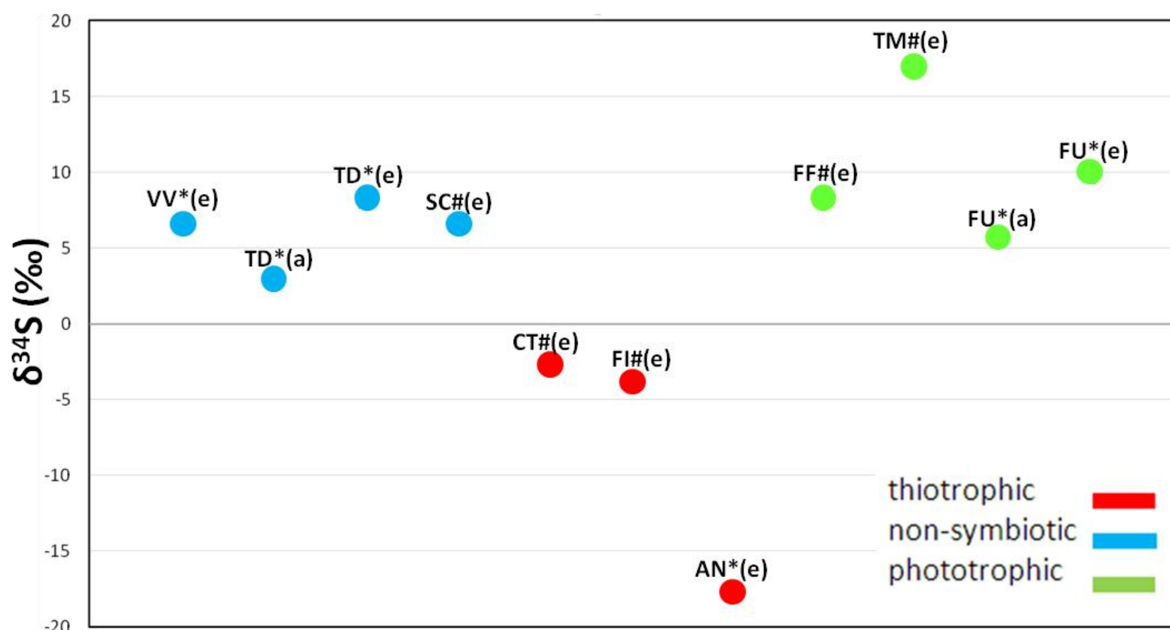

Fig. 2. $\delta^{34}S$ of organic sulfur in bulk organic shell matrix from shells of living bivalves (a), and empty shells (e). Type of lifestyle is color coded: (red) thiotrophic, (blue) non-symbiotic, (green) phototrophic. Abbreviations according to Table 1. (*= North Stradbroke Island, #= One Tree Island). Measurement inaccuracy: $\delta^{34}S$ ±0.3 ‰.

Lipids

The $\delta^{13}C$ values of fatty acids of living bivalve shells and sampled empty shells are shown in Table 2. Compounds in the neutral lipid fraction were very low in abundance and were too low for robust identifications (and $\delta^{13}C$ analyses; data are therefore not shown). Compositions of fatty acids in empty shells and living bivalve shells were nearly similar (Fig. 3). Hexadecanoic and octadecanoic acid and their unsaturated homologues were prominent in all samples. Tetradecanoic acid and terminally branched (*iso* and *anteiso*) pentadecanoic acids, however, were only found in empty shells. A broad range of $\delta^{13}C$ values of fatty acids was detected (Table 2). The $\delta^{13}C$ values from lipids of the (empty shell) non-symbiotic *Scutarcopagia sp.* from One Tree Island were less depleted in ^{13}C than the lipids from thio- and phototrophic bivalves from the same setting. Eight of ten lipids from phototrophic *F. fragum* showed less ^{13}C depletion than lipids from the other symbiotic species. The strongest depletion was observed for lipids of *T. maxima* and *F. fimbriata* (Table 2).

Table 2. Total lipid $\delta^{13}C$ values (‰ relative to VPDB) for empty shells of bivalves from One Tree Island and with *Fragum unedo* from North Stradbroke Island.

Selected Lipids	*C. tigerina* (modern empty shell) $\delta^{13}C$ (‰)	*F. fimbriata* (modern empty shell) $\delta^{13}C$ (‰)	*F. fragum* (modern empty shell) $\delta^{13}C$ (‰)	*T. maxima* (modern, empty shell) $\delta^{13}C$ (‰)	*T. maxima* (fossil, empty shell) $\delta^{13}C$ (‰)	*F. unedo* (modern live shell) $\delta^{13}C$ (‰)	*Scutarcopagia sp.* (modern empty shell) $\delta^{13}C$ (‰)
Tetradecanoic acid	-20.2	-25.9	-18.1	-27.5	n.a.	n.d.	-11.8
13-Methyltetradecanoic acid (iso)	-20.7	-27.2	-14.8	-20.9	n.a.	n.d.	n.d.
12-Methyltetradecanoic acid (anteiso)	-23.2(1)	-27.7	-18.8(1)	-26.9	n.a.	n.d.	n.d.
Pentadecanoic acid	-23.3	-23.8	-18.1	-25.6	n.a.	n.d.	-10.2
Palmitoleic acid: 16:1ω5c	-17.3	n.d.	-16.6	-25.4	n.a.	n.d.	-9.7
Hexadecanoic acid	-20.1	-23.5	-20.5	-25.5	n.a.	-28.2	-13.2
Heptadecanoic acid	-24.4(1)	-24.5	-22.3	-24.4	n.a.	n.d.	n.d.
Oleic acid: 18:1ω9c	-23.2(1)	-25.5	-18.4	-27.7	n.a.	n.d.	n.d.
Oleic acid: 18:1ω7c	-16.7(1)	-23.9	-17.1	-23.5	n.a.	n.d.	n.d.
Octadecanoic acid	-24.9	-26.7	-23.5	-25.3	n.a.	-29.5	n.d.

Standard deviations of replicate measurements were largely < 0.5 ‰ and sometimes < 1 ‰. n.d., no data, n.a. not analyzed.

Table 3. $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ ratios of bulk organic shell matrix from the shells of two living individuals and empty shells. Carbon isotopic compositions are reported relative to V-PDB. Nitrogen is reported relative to the atmospheric air standard and sulfur is reported relative to CDT standard.

	Sample	Isotopic composition			
		$\delta^{13}C$ (‰) ±0.1‰	$\delta^{15}N$ (‰) ±0.2‰	$\delta^{34}S$ (‰) ±0.3‰	C/N-ratio
thiotrophic	*Codakia tigerina* (One Tree Island)	-26.2	-1.2	-2.5	4.06
	Fimbria fimbriata (One Tree Island)	-30.6	-2.2	-3.9	3.5
	Anodontia sp. (Moreton Bay)	-25.5	+0.1	-17.9	3.74
filter feeder	*Vasticardium vertebratum* (Moreton Bay)	-21.98	+3.1	+6.7	3.1
	Tapes dorsatus (Moreton Bay)	-20.10	+4.2	+8.0	3.21
	Tapes dorsatus (Moreton Bay.,alive)	-17.8	+4.3	+2.7	3.08
	Scutarcopagia sp. (One Tree Island)	-20.6	+3.2	+6.4	3.76
phototrophic	*Fragum unedo* (Moreton Bay)	-28.69	+2.5	+9.7	3.78
	Fragum unedo (Moreton Bay., alive)	-26.0	+2.9	+5.9	3.61
	Tridacna maxima (One Tree I.sland)	-21.4	+1.6	+16.8	3.47
	Tridacna maxima (fossil, Sinai, Egypt)	n.d.	n.d.	n.d.	n.d.
	Fragum fragum (One Tree Island)	-31.0	+1.7	+8.2	4.21

n.d. = no data

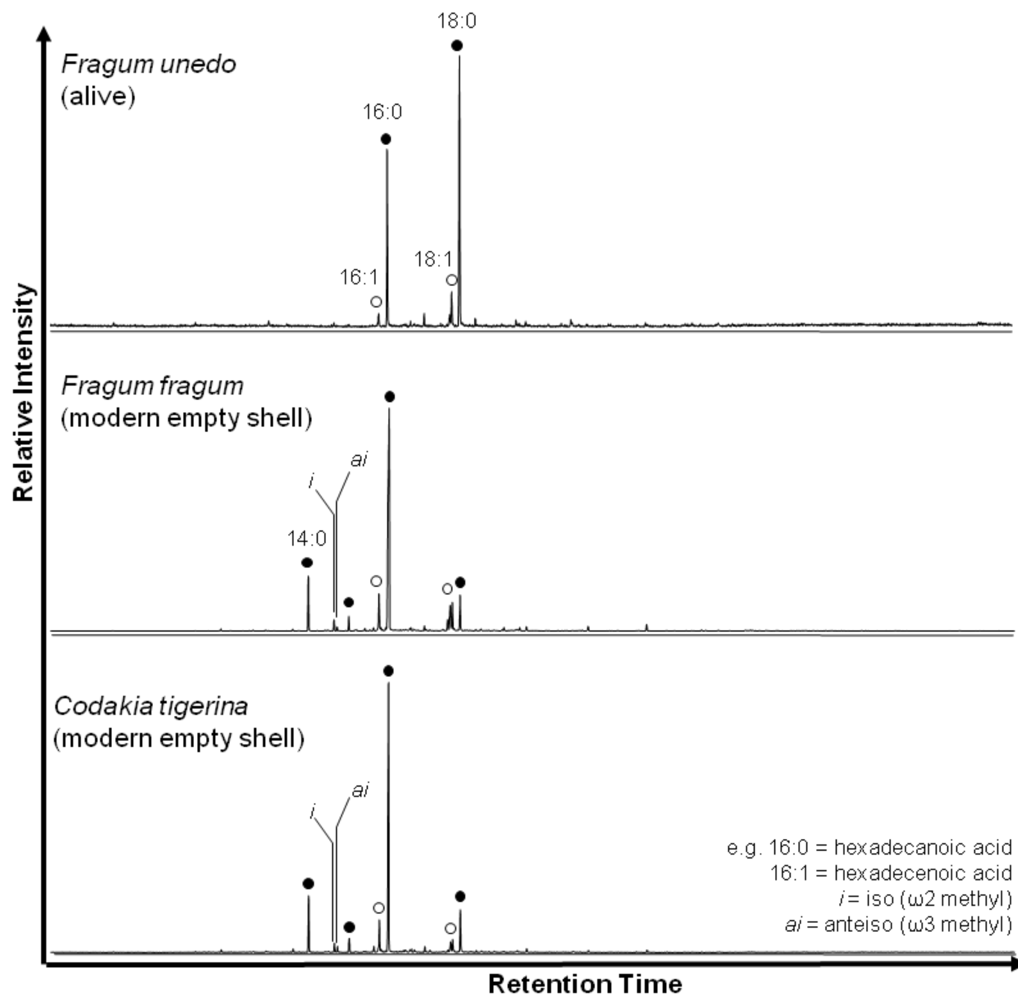

Fig. 3. Total ion chromatograms (TICs) of fatty acid compositions, extracted from the shell of modern live phototrophic *Fragum unedo*, modern empty phototrophic *Fragum fragum* and modern empty thiotrophic *Codakia tigerina*. Filled circles denote saturated fatty acids and open circles unsaturated fatty acids.

4.4.3 Microscopic analyses of a modern empty and a fossil Tridacna maxima shell.

To determine a possible quality loss of bulk organic shell matrix, a modern (empty shell) and fossil *T. maxima* shell were visually analyzed by light and electron microscopy. The EDTA eroded surfaces of pieces from fossil and recent *T. maxima* shell were analyzed by SEM. Figure 4 and 5 show details of a shell piece from modern *T. maxima.* In Figure 4 (A) a smooth (soft) substance is visible lying on parts of crossed lamellar layered mineral from modern shell

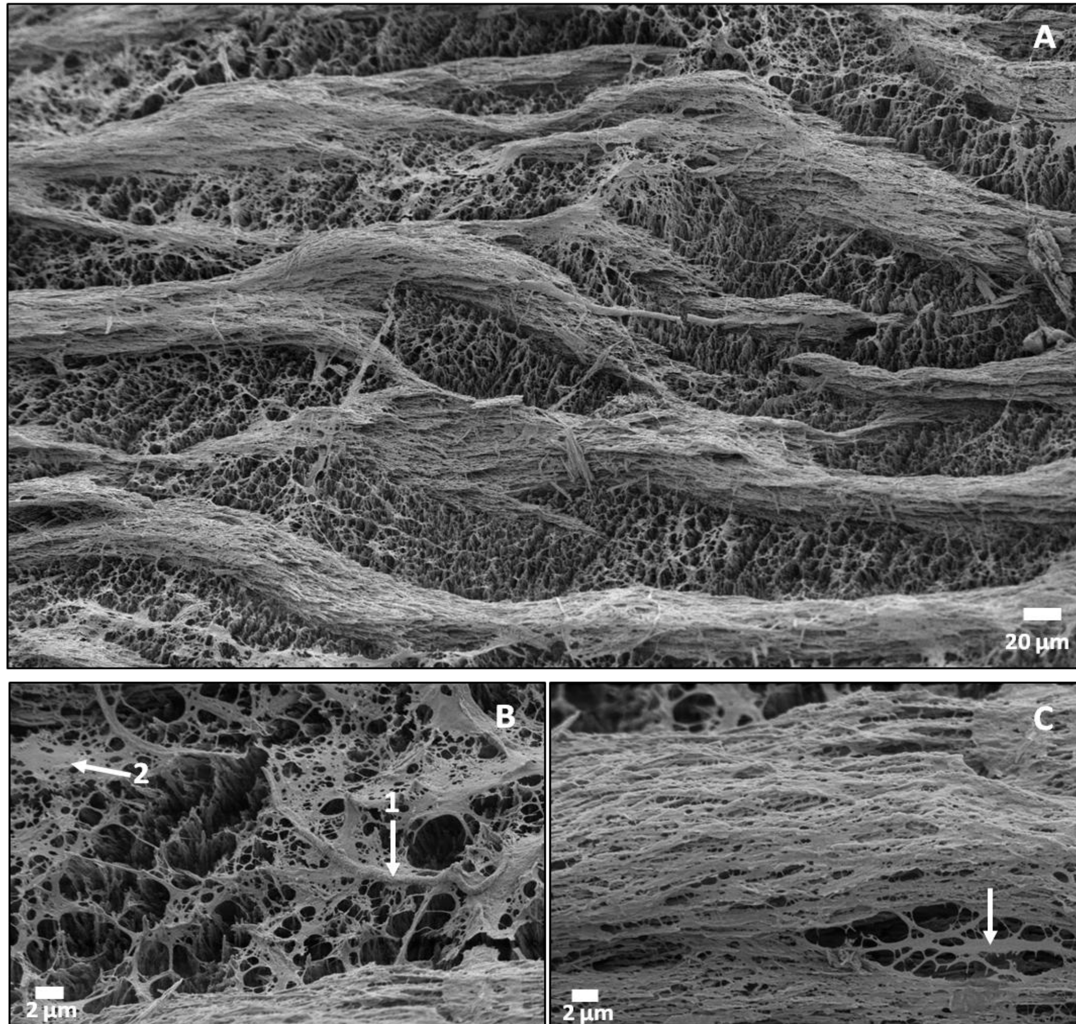

Fig. 4. Scanning electron micrograph of EDTA eroded surface of a modern *Tridacna maxima* shell. A) General view of the shell surface, the crossed lamellar structure is visible. B) Higher magnification of A, filamentous microorganisms (arrow1) within the organic matrix (arrow 2) C) Higher magnification of A, the organic shell matrix spanning over mineral structures (arrow)

sample. At higher magnification the smooth substance displayed a web-like structure covering the mineral phase of the shell (Fig. 4C arrow, 4B arrow 2). Additional sheets and filaments (flatted due to dehydration) of microorganisms could be found within the smooth substance (Fig. 4B arrow 1) or isolated at the EDTA eroded mineral surface (Fig. 5). In contrast to the modern shell the corroded surface from fossil *T. maxima* shell did not show the smooth substance (Fig. 6A and B); only the crossed lamellar structure of the shell was visible. The evaluation of the completely decalcified remaining residue from

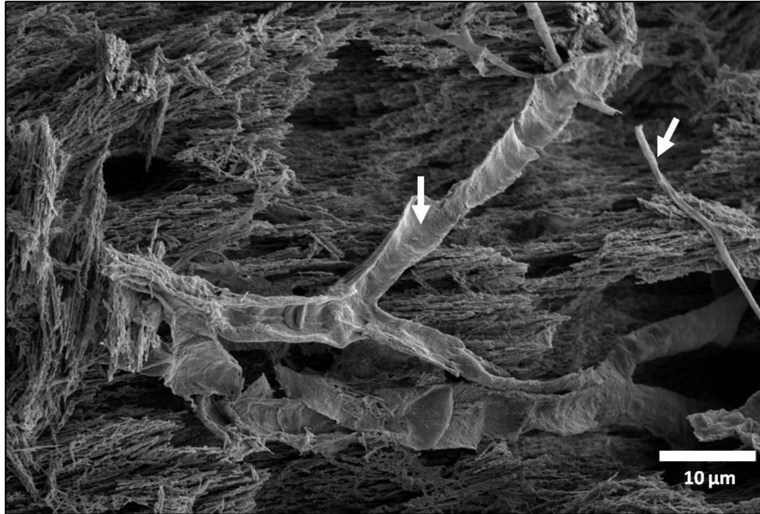

Fig. 5. Scanning electron micrograph of EDTA eroded surface of a modern *Tridacna maxima* shell. Filamentous microorganisms are observed (arrows).

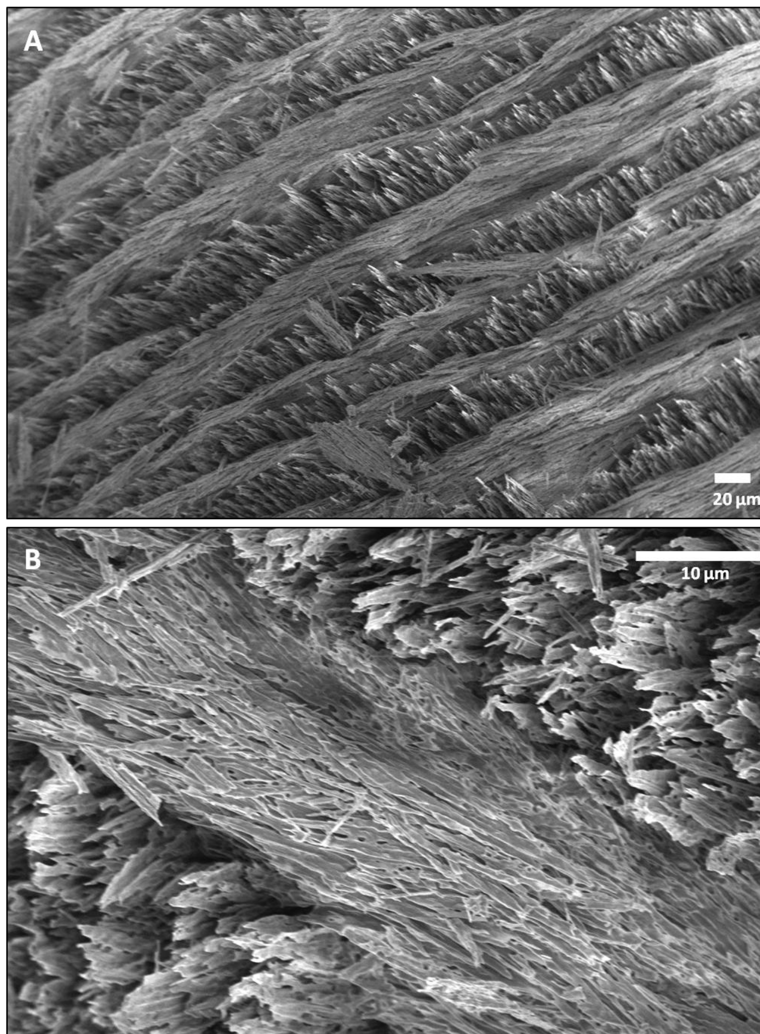

Fig. 6. Scanning electron micrograph of EDTA eroded surface of a fossil (Pleistocene) *Tridacna maxima* shell. A) General view of the shell surface, the crossed lamellar structure is visible. B) Higher magnification of A, no smooth organic shell matrix is visible.

modern and fossil *T. maxima* shell pieces indicates a clear difference between both types of samples. The former appeared more voluminous and had a white color whereas the latter, fossil residue was flat and brownish in color (visual observation). Light microscopic analysis of modern residue showed a fibrous web-like material stained by Calcofluor white (Fig 7A and B). The fibrous web-like material was not observed in the brown organic residue from the fossil shell piece stained with Calcofluor white (Fig. 7C). Also, sheaths of filamentous microorganisms are visible in modern *T. maxima* organic residue (Fig. 8A and B). Similar filamentous sheet-like structures were observed in fossil *T. maxima* (Fig. 9B arrow), besides or partially within these sheaths-filamentous structures dark spheres were visible (Fig. 9A arrow and B). These spheres were also observed with SEM (Fig 10A and B) and EDX analyses revealed that they consisted of iron sulfide (data not shown).

Within some parts of the thin sections of embedded and decalcified modern *T. maxima* shell, a group of morphologically-varied filamentous organisms within the shell were observed. These organisms show a strong auto-fluorescence signal (Fig. 11A - B).

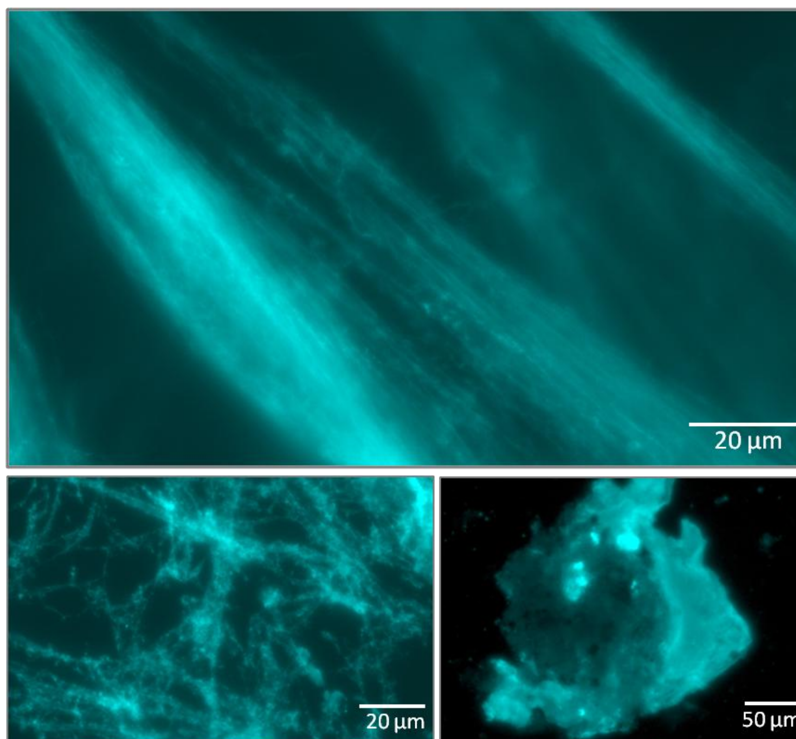

Fig. 7. Chitin staining with Calcofluor White of completely decalcified *Tridacna maxima* shell piece. A) and B) Modern shell, organic flake, note the fibrous web-like material. C) Fossil shell (Pleistocene); the fibrous material absent. (modified after Dreier & Hoppert, 2013 in press)

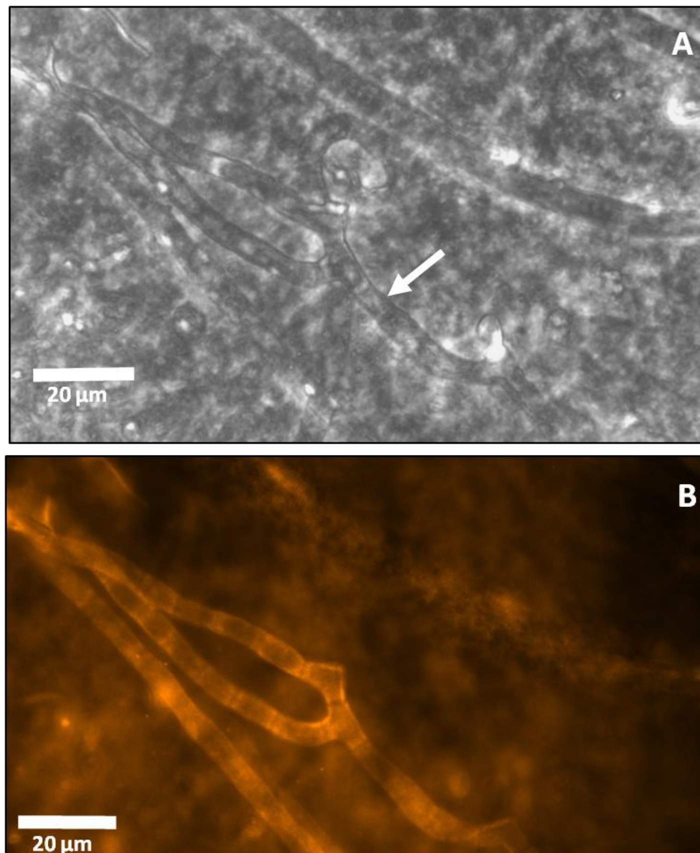

Fig. 8. Microscopic image of completely decalcified piece of a modern shell from *Tridacna maxima.* A) Phase contrast image of filamentous microorganisms within the organic residue. B) Same picture as A, note the auto-fluorescence of the filamentous microorganisms.

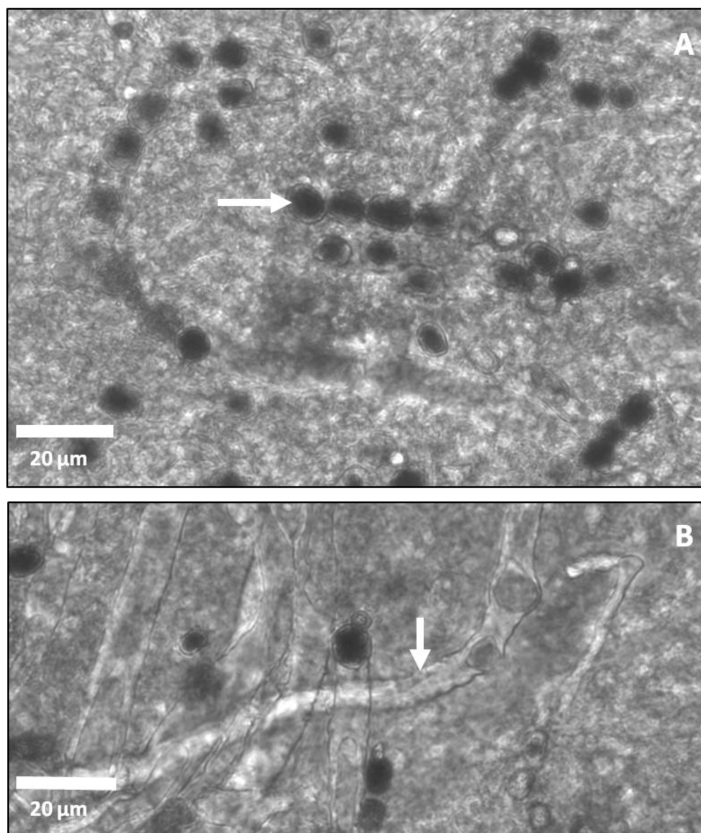

Fig. 9. Phase contrast image of completely decalcified piece of fossil shell from *Tridacna maxima.* A) Framboidal pyrite spheres (arrow) are visible. B) Empty filamentous sheet (arrow).

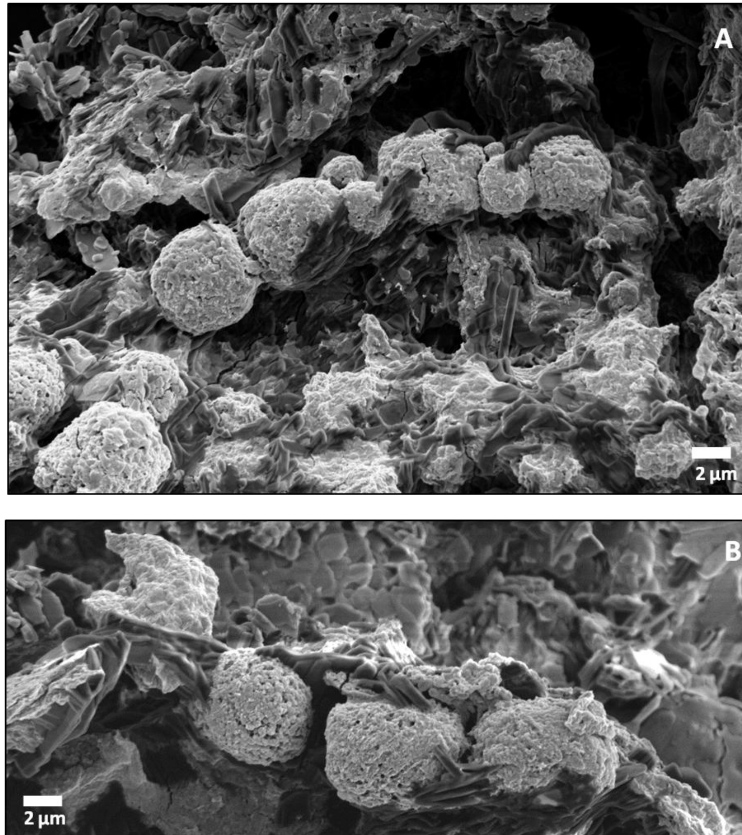

Fig. 10. Scanning electron micrograph of completely decalcified piece of fossil shell from *Tridacna maxima.* A), B) Note the framboidal pyrite spheres.

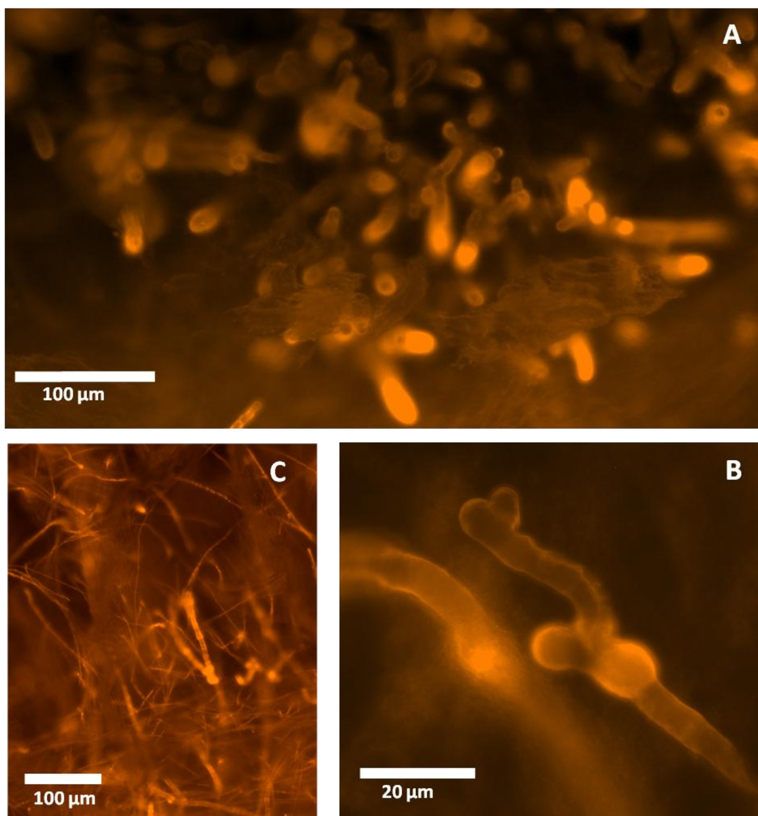

Fig. 11. Epifluorescence micrographs of ca. 300 µm thin sections from embedded and decalcified shell of modern *Tridacna maxima*. A), B) and C) different types of filamentous microorganisms showing a high auto-fluorescence.

4.5 Discussion

4.5.1 Symbionts of investigated bivalves

16S rRNA and ITS2 gene sequence analysis confirmed the presence of microbial symbionts in the investigated bivalves. The symbiont of *Anodontia sp.* was identified as a sulfur oxidizing gammaproteobacterium. Up to now, very little is known about the phylogenetic affiliation of the *Fimbria*-chemosymbiont (Janssen, 1992). Sequence analysis in our study showed 97 % similarity to an uncultivated gammaproteobacterium. In our study no sequence could be amplified from *C. tigerina* gill tissue, possibly due to PCR inhibition or degradation of sample DNA (only one individual of living *C. tigerina* could be collected). Symbionts of this species, however, have been already extensively studied and were reported to be comprised of sulfur-oxidizing bacteria affiliated to gammaproteobacteria (Frenkiel & Mouëza, 1995; Durand et al., 1996). Phototrophic endosymbionts of *Tridacna spp.* and *Fragum spp.* have been identified as *Symbiodinium spp.* algae. This could be confirmed in this study. Symbionts of both *Fragum* species could be affiliated identified to clade C phylotypes of *Symbiodinium*.

4.5.2 Carbon and nitrogen isotope signatures

Bulk organic shell matrix

Bivalves harboring phototrophic or chemotrophic (thiotrophic) symbionts are often also capable of filter-feeding (Hawkins & Klumpp, 1995; Duplessis *et al.,* 2004), but mainly cover their energy demands through the metabolism of their symbionts (Lebata & Primavera, 2001; Duperon et al., 2007). Thus, if the majority of carbon and nitrogen is derived from symbionts, the isotopic signatures of host tissue (bulk organic shell matrix) should reflect autotrophic and /or nitrogen metabolism of these microorganisms (Trench et al., 1981; Herry & LePennec, 1989; Klumpp et al., 1992; Dreier et al., 2012; Vafeiadou et al., 2013).

The $\delta^{13}C$ of bulk organic shell matrix from the studied thio- and phototrophic shells exhibited values ranging from -25.5 ‰ to -31.0 ‰ (Tab. 3, Fig. 1; including modern living and empty shells). While non-symbiotic bivalves

revealed the highest $\delta^{13}C$ values, no difference was observed between carbon isotopy of photo- vs. thiotrophic bivalves.

An important factor affecting carbon isotope signatures is the form of the RubisCO protein which is used by the symbionts for CO_2 fixation. Tissue from invertebrates with symbionts using form I RubisCO was found to be more depleted in ^{13}C than that of invertebrates with symbionts using form II RubisCO (Childress & Fisher, 1992; Van Dover & Fry, 1994; Robinson & Cavanaugh, 1995; Cavanaugh & Robinson, 1996; Robinson et al., 2003). No reports exist on the form of RubisCO used by the thiotrophic lucinids *F. fimbriata* and *C. tigerina*, but a form I RubisCO was detected in symbionts from other thiotrophic lucinid bivalves (Cavanaugh & Robinson, 1996) which may also be anticipated for the thiotrophic lucinids of this study. For the phototrophic *Fragum*, it is well known that its symbiont *Symbiodinium sp.* uses a form II RubisCO to fix CO_2 (Rowan et al., 1996). However, our data revealed no ^{13}C enrichment of *Fragum spp.* as compared to the thiotrophic lucinids (Fig. 1). It will be necessary to determine each RubisCO form used by the individual *F. fimbriata* and *C. tigerina* symbionts, to confirm whether the particular type of RubisCO is an important determinant of the $\delta^{13}C$ from these symbiont-bearing invertebrates.

The $\delta^{13}C$ values of bulk organic shell matrix from both phototrophic *Fragum* species range from -26.0 ‰ to -31.0 ‰ respectively, with *F. fragum* showing the lower value. *F. fragum* were sampled from the sandy substrates of a coral reef ecosystem where nutrient-limited conditions are common. The lower values suggests that *F. fragum* depends more on symbiont-fixed carbon than *F. unedo* which were collected from nutrient-rich seagrass beds of Moreton Bay. Also, the $\delta^{15}N$ value of *F. fragum* was lower (1.7 ‰) than *F. unedo* (2.9 ‰ and 2.5 ‰), supporting the assumption that *F. fragum* has incorporated a higher proportion of symbiont-derived metabolites.

In contrast to the phototrophic *Fragum* species, the $\delta^{13}C$ value from the phototrophic *T. maxima* of -21.4 ‰ was relatively higher. The *T. maxima* shell-valve used for this study was small (approximately 12 cm in length), and originated from a relatively young individual. Hawkins & Klumpp (1995) reported a greater dependence on filter-feeding for small *T. gigas* whereas older and larger-sized clams rely more on their endosymbiotic *Symbiodinium* to meet their

carbon needs. Such greater contribution of organic carbon acquired by filter-feeding may plausibly explain the relatively higher $\delta^{13}C$ value observed for the young *T. maxima* in our study. In addition, microorganisms found within the modern shell of *T. maxima* may have produced a shift of $\delta^{13}C$ values and started degradation of the bulk organic shell matrix (see discussion below). However, given the much higher proportion of bulk organic shell matrix biomass compared to the microbial biomass observed within the shell (personal observation), the striking difference in $\delta^{13}C$ values (9.6 ‰) between phototrophic *F. fragum* and *T. maxima* from the same sampling site cannot be fully be explained by the addition of organic matter from microbial contaminants, but may rather be related to the predominantly filter-feeding lifestyle of young *T. maxima*.

The comparison of $\delta^{13}C$ values between bulk organic shell matrices from living and empty shells of the same species, showed a shift of around 2.5 ‰. This shift may be due to bulk organic shell matrix degradation or could be attributed to different environmental conditions during the lifetime of the bivalve. The former assumption was preferred, as preliminary results of a taphonomy experiment, show a slight decrease of $\delta^{13}C$ values from bulk organic shell matrices after half a year in sediment following death (unpublished data).

For the accurate determination of bivalve diet/lifestyle, it is important that isotopic differences between species remain consistent and stable. This seems to be the case here because shells from both species (*F. unedo* and *T. dorsatus*) show nearly the same shift after death, so that the difference of isotopic values was not affected (Fig. 1, Table 3).

Interestingly, of the three thiotrophic, members of the family of Lucinidae, exhibit consistently lower $\delta^{15}N$ values (down to -2.2 ‰ for *F. fimbriata*) than the other bivalves studied. This seems to be a common isotopic trait of the Lucinidae, as low $\delta^{15}N$ values of lucinid tissue in comparison to other bivalves from same sampling site were also observed in the study of Dreier et al. (2012) and Vafeiadou et al. (2013).

It is reported that $\delta^{15}N$ ratios increase by about 3.4 ‰ per trophic level (Minagawa & Wada, 1984). In this study we presume that phototrophic and thiotrophic bivalves are at the same trophic level. Thus, the observed difference

in $\delta^{15}N$ values could not exclusively be explained by placement (level) in the food chain.

Dreier (et al., 2012) showed that bacterial glutamine synthetase (GSI) was expressed by endosymbionts in the lucinid *Loripes lacteus*. It was hypothesized that these symbionts are responsible for assimilation of ammonium, and linked to the reduction of *Loripes* shell $\delta^{15}N$ values. Eukaryotic glutamine synthetase (GSII) may also be expressed by the photosymbionts of bivalves. Indeed, significant ammonium assimilation was attributed to *Symbiodinium* from phototrophic bivalves such as *T. gigas* (Anderson & Burris, 1987) and various cnidarians (Hawkins & Klumpp, 1995), and may also occur with the *Fragum spp.* studied. In contrast to bacterial symbionts of lucinid bivalves, *Symbiodinium* GSII may account for the $\delta^{15}N$ differences between thiotrophs and phototrophs. But the striking difference between $\delta^{15}N$ values of thiotrophic bivalve tissue may not originate from different fractionation pattern of GSI and GSII alone. It is known that in bacteria, the uptake of ammonium across the membrane leads to additional ^{15}N depletion (Hoch et al., 1992). Differences in uptake mechanism of ammonium by bacteria and *Symbiodinium* may explain the observed fractionation pattern.

Another explanation for the reduced $\delta^{15}N$ values in thiotrophic bivalves compared to phototrophic and non-symbiotic is based on the ability for nitrate respiration among thiotrophic symbionts (Wilmot and Vetter, 1992; Hentschel et al., 1996). A fractionation of nitrogen isotopes during first step of nitrate respiration (denitrification) was described (Knöller et al., 2011). Thus, the product nitrite should be depleted in ^{15}N. The depleted nitrite could end up in the assimilatory pathway of nitrate reduction. The existence of the assimilatory nitrate reductase pathway in some chemosymbiontic bivalves was proposed by Lee & Childress, (1994).

Except *V. vertebratum*, the $\delta^{15}N$ values of phototrophic bivalves in this study including *T. maxima* are generally lower than in non-symbiotic bivalves (Fig. 1) by approximately 1 ‰ with respect to sampling site.

Lipids (fatty acids)

Lipids are only a minor constituent of shell organic matter of bivalves (Wilbur & Simkiss, 1968; Hare & Hoering, 1977; Weiner, 1988; Lowenstam & Weiner, 1989). However, in living bivalves fatty acids appear to be useful for the distinction of a thiotrophic versus a non-symbiotic lifestyle of bivalves (Dreier et al., 2012). Whether this is also applicable for empty sampled shells was part of the current study.

No distinctive pattern of $\delta^{13}C$ values was observed for selected shell-lipids from phototrophic *vs.* thiotrophic bivalves from the same sampling site (Table 2). Interestingly, most lipids of *F. fragum* were less depleted in ^{13}C than in the other symbiotic bivalves, whereas the opposite was observed for $\delta^{13}C$ values from bulk organic shell matrix. A consistent difference was observed between the lipids of phototrophic *F. fragum* and thiotrophic *F. fimbriata,* at which the lipids of *F. fimbriata* show a higher ^{13}C depletion (Table 2). In contrast, the measured $\delta^{13}C$ of bulk organic shell matrix from *F. fragum* and *F. fimbriata* are nearly identical (-31.0 ‰ and -30.6 ‰, respectively; Tab. 3). Typically, lipids are isotopically lighter than other biochemical fractions like organic shell matrix (Parker 1964; DeNiro & Epstein 1977; Pinnegar & Polunin 1999), a relationship which is not dependent on the lifestyle of the organisms. In our study this was only the case for lipids from a living *F. unedo* shell, the only one for which the whole data set was available, and for lipids from the *T. maxima* shell which were more depleted than the measured $\delta^{13}C$ ratio of -21.4 ‰ from *T. maxima* bulk organic shell matrix. However, results from *T. maxima s*hell are questionable, because this shell likely contained biomass of boring microorganisms (see below).

$\Delta^{13}C$ values of fatty acids from shells of living *F. unedo* are consistent with previous reports on fatty acids from living bivalves shells (Dreier et al., 2012) and the general biosynthetic biomass-lipid isotopic relationship (Hayes, 2001). The distribution of fatty acids (mostly unspecific) was similar in living and empty shells exept that *iso* and *anteiso* pentadecanoic acid (*i*15:0 and *ai*15:0) were detected only in empty shells. Invading prokaryotes are most likely the major source of the extra and possibly some other unspecific fatty acids found in empty shells. This is evident by (i) the $\delta^{13}C$ values of fatty acids, and (ii) the

presence of the bacterial fatty acid *anteiso* pentadecanoic acid (ai15:0), which is in marine settings specific for sulfate reducing bacteria (Boon et al., 1977; Dowling et al., 1986). Our fatty acid data therefore point to a low preservation potential for bivalve fatty acids in the shell (or on the shell surface) and argue against their applicability as biosignatures in ancient bivalves.

4.5.3 Sulfur isotope signatures

In a precious study (Dreier et al., 2012) we showed that the $\delta^{34}S$ values of bulk organic shell matrices (of live shells) were negative for thiotrophic but positive for non-symbiotic bivalves. It was thus proposed that the sulfur isotopic composition of the bulk organic shell matrix can be used as biosignature for a thiotrophic lifestyle of a bivalve. However, it could also be shown that $\delta^{34}S$ values were not stable and decreases after the death of the bivalve. In the present study the isotopic composition of bulk organic shell matrices were determined mostly from empty shells. In accordance with the thiotrophic bivalves analyzed in Dreier et al. (2012), all $\delta^{34}S$ values determined here for thiotrophic bivalves were negative (Fig. 2). No significant differences were observed between $\delta^{34}S$ values of non-symbiotic and phototrophic bivalves, with both groups showing positive values for, samples from either living animals or from empty shells (Fig. 2). For empty shells it may be speculated that the time point of death was recent and the resting time within the sediment was too short for to destabilize and lower the $\delta^{34}S$ values as observed by Dreier et al. (2012). Despite the clear ^{34}S depletion observed for empty thiotrophic shells, negative $\delta^{34}S$ values are not a robust signature for thiotrophic lifestyle due to diagenetic overprint, while positive values appear to be good indicator for non-symbiotic and phototrophic lifestyles. This suggests that positive $\delta^{34}S$ values of bulk organic shell matrix may be used as a biosignatures to exclude the possibility of a thiotrophic lifestyle for a bivalve.

4.5.4 Quality of bulk organic shell matrix from a modern empty shell vs. a fossil shell of T. maxima

The aim of this study was to identify isotopic signatures in bulk organic shell matrices of bivalves to differentiate between thiotrophic, phototrophic and non-

symbiotic lifestyles. Our data may also help to evaluate how useful and reliable these traits are for the determination of bivalves lifestyles based on shell residues of all kind. Though the bulk organic shell matrix may be stable over certain period of time, it was not possible to extract a sufficient amount of bulk organic shell matrix from a fossil (late Pleistocene) *T. maxima* shell for isotope analysis. Compared with an extract obtained from a modern empty *T. maxima* shell of the same size, the organic extracts also differed qualitatively. The remaining bulk organic shell matrix of the modern shell appeared thickened 'jelly-like' and showed a pale yellowish color whereas the fossil residue appeared brownish and flattened. Microscopic analysis showed that the bulk organic shell matrix of modern *T. maxima* shell has a smooth fibrous web like structure (Fig. 7A and B). These fibrous matrices were stained by Calcofluor white (Fig. 7A and B) indicating polysaccharides such as chitin which is a common material of mollusk shells (Degens et al., 1967; Jeuniaux, 1963; Weiner and Traub, 1980, Launspach et al., 2012).

Inside the fibrous material (of modern empty shell pieces) filamentous microorganisms were observed (Fig 8A and B). These filaments were also observed by SEM at the EDTA eroded surface of modern *T. maxima* shell pieces (Fig. 5 arrows). To confirm that the observed microorganisms were not artifacts from the outer shell surface, thin sections of the modern shell (decalcified with EDTA) were also analyzed microscopically (Fig 11A - C). Filamentous microorganisms were found within the shell of *T. maxima* and showed a strong autofluorescence. Endolithic microorganisms boring in carbonates shells and substrates are widespread in marine environments; fungi, microalgae and cyanobacteria have been described to date (Golubic, 1969; Burford et al., 2003; Hoppert et al., 2004). Some filaments could be found directly within the smooth substance (putative bulk organic shell matrix, Fig. 4B arrow 1). In former studies it was observed for endolithic fungi that they grow inside layers of bulk organic shell matrix (Golubic et al., 2005). However, the filaments we observed here were not stained by Calcofluor white which normally binds to fungal cell walls. Due to their morphology it may be assumed that most of the microorganisms found represent boring cyanobacteria (compare with e.g. Günther, 1990, Chazottes et al., 2009).

At the EDTA eroded surface of fossil and modern *T. maxima* shell pieces, the cross lamellar mineral structure is visible (Fig. 4A and 6A). The smooth bulk organic shell matrix covering the actual mineral was only present in the modern shell (Fig 4C arrow, 4B arrow 2), whereas no organic shell matrix was visible in fossil shell preparation (Fig. 6A and B).
The material left from the fossil shell looks different, no fibres could be found only “flakes” (Fig. 7C) and empty sheaths and numerous of dark spherical particles are preserved (Fig 9A and B). The empty sheaths have nearly the same size like those found in modern *T. maxima* shell (Fig 8A arrow). This indicates that the fossil *T. maxima* shell was also “infected” by boring microorganisms. The morphology and EDX spectra (high content of iron and sulfur, data not shown) of the spheres observed by SEM, confirm that these spheres are framboidal pyrite (Fig. 10A and B; Berner, 1984; Wilkin, 1995). The abundance of this framboids within the fossil shell suggests former/ancient activity of different microbes, particularly by sulphate-reducing bacteria inside the shell (Goldhaber & Kaplan, 1974; Beveridge et al., 1983; Kohn et al., 1998).
Our results show that, after death of a bivalve, the organics contained within its shell was subjected to a gradual degradation and transformation. This degradation may be observed microscopically and indicated by shifts of fatty acid composition. However, the bulk organic shell matrix seems to be more resistant to the early diagenetic processes but disappear during longer times.

4.6 Conclusion

This study shows that $\delta^{34}S$ values of bulk organic shell matrix from empty shells could be helpful to exclude a thiotrophic lifestyle of both modern and fossil bivalves. Whenever the bulk organic shell matrix exhibits positive $\delta^{34}S$ values, it belongs to non-symbiotic or phototrophic bivalves. However, negative $\delta^{34}S$ values do not necessarily indicate thiotrophic symbiosis (see Dreier et al., 2012).
The $\delta^{13}C$ ratio of bulk organic shell matrix seems to be useful to distinguish between non-symbiotic (higher $\delta^{13}C$ values) and symbiotic (lower $\delta^{13}C$ values)

lifestyle, whereas no clear differentiation between phototrophic and thiotrophic symbiosis is possible.

$\delta^{15}N$ values in combination with the $\delta^{13}C$ values from bulk organic shell matrix are useful signatures to discriminate between non-symbiotic, phototrophic and thiotrophic symbiosis in bivalves from the same sampling site.

During early diagenesis, slight changes of lipid composition between alive *v.* modern empty shells indicate a microbial origin for the latter.

A substantial loss of bulk organic shell matrix was observed in the fossil *T. maxima* shell, indicating a degradation of shell organics during fossilization in some cases. Additional contamination with boring microbes was observed in the modern empty *T. maxima* shell. However, by using $\delta^{13}C$ and $\delta^{15}N$ of the remaining bulk organic shell matrix it is possible to reconstruct the lifestyle of bivalves from modern empty shells and, under optimal conditions, from subfossil shells (Dreier et al., 2012). Hence, the quality of the bulk organic shell matrix should be evaluated (e.g. via microscopic techniques) before determining the isotopic signatures.

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5. General Discussion and Conclusion

In the previous chapters the identification of biochemical markers within mollusk shells, suitable for the determination of symbiotic associations, was described. Further, the stability of these markers over geological times was evaluated with the aim to improve knowledge on putative symbiotic lifestyles of ancient (fossil) bivalve species. Mollusk shell-organics are primarily composed of proteins and polysaccharides, including amino sugars (e.g. Weiner et al., 1982); also a small fraction of lipids was found (Beedham 1958). In this study, the mineral phase of the shell was dissolved and the isotopic composition of organic carbon and nitrogen was analyzed.

For this purpose a non-symbiotic bivalve species (*Venerupis aurea*) and a bivalve species (*Loripes lacteus*) which harbors chemoautotrophic bacteria served as a model system representing two different lifestyles (see Chapter 3).

In contrast to the filter-feeding *Venerupis* which takes up heterogeneous organic particles from environment, *Loripes* covers its major nutrient demand via biomolecules from its symbionts (Cavanaugh et al., 2006; Dubilier et al., 2008).

The study presented here is based on the assumption, that the metabolism of thiotrophic symbionts in *Loripes* causes a characteristic isotopic fingerprint within the organic shell matrix. It was shown in Chapter 3 that symbionts of *Loripes* use the Calvin-Benson cycle to fix carbon. In addition, it was shown that the symbionts are responsible for the incorporation of ammonia into amino acids. Both bacterial metabolic pathways lead to a fractionation of either carbon or nitrogen isotopes within bacterial biomolecules (Ruby et al., 1987; Hoch et al., 1992; Yoneyama et al., 1993; Purich & Allison, 2000; Blumenberg, 2010).

Thus, the isotopic composition of host biomolecules within the shell of *Loripes* clearly reflects the symbiont-based diet. The $\delta^{13}C$ of shell-lipids as well as of bulk organic shell matrix was found to be much lower in the symbiotic *Loripes* than in the filter-feeding *Venerupis*. Likewise the bulk organic shell matrix of *Loripes* was more depleted in ^{15}N than in *Venerupis* (see Chapter 3).

It turned out that the composition of shell-lipids in preparations from living samples differs from lipids extracted from empty shells (see Chapter 3 and 4). In empty shells tetradecanoic acid and terminally branched (*iso* and *anteiso*) pentadecanoic acids were found; these short chained fatty acids could also be

found in sediment (Cobabe & Pratt, 1995). *Anteiso* pentadecanoic acid indicates the presence of sulfate reducing bacteria (see Chapter 4). In addition, framboidal pyrite was found in a fossil shell of *Tridacna maxima,* a further trace of microbial sulfate reduction (Chapter 4) and of the possible involvement of sulfate reducers in degradation of shell-organics.

Lipids of definite host origin e.g. cholesterol or docosadienoic acid were only detected in living shells of *Loripes* and *Venerupis* (Chapter 3). For some unknown reason they were not found in the living shell of *Fragum unedo* (Chapter 4) and they were absent from all empty shells analyzed in this study. Hence, in non-living samples, lipids extracted from shell rather derive from bacterial destruents than from the bivalve itself.

Previous studies showed that lipids were generally more depleted than e.g. proteins or sugars (Parker 1964; DeNiro & Epstein 1977; Pinnegar & Polunin 1999). This was observed here only for shells from bivalves sampled alive but not for empty shells, further supporting the assumption that the extracted lipids did not derive from bivalve tissue.

Nevertheless, the differences of lipid $\delta^{13}C$ values between symbiotic and non-symbiotic lifestyles were also observed for empty shells (see Chapter 4). This may indicate that lipids, in fact, derive from bacteria but that the food sources of those bacteria are based on shell -organics. Thus, the differences in lipid $\delta^{13}C$ values remain stable between two lifestyles, while absolute values are shifting.

Shell-lipids represent only a very minor component of shell-organics. Proteins and polysaccharides turned out to be more tightly bound to shell -minerals during early diagenesis than lipids and were the major source of organic carbon and nitrogen. Thus, $\delta^{13}C$ and $\delta^{15}N$ values of bulk organic shell matrix from a subfossil *Loripes lacteus* and *Venerupis aurea* revealed that the different depletion patterns of symbiotic vs. non-symbiotic lifestyle was also stable over time (see Chapter 3).

Based on these results the study was expanded with respect to distinction between thiotrophic and “phototrophic” bivalve shells (see Chapter 4). The $\delta^{13}C$ signature of shell-lipids is not useful, because it seems that lipids of host origin get lost after death of the bivalve. Moreover, it is not possible to distinguish

between thiotrophic and phototrophic symbiosis on the basis of lipid $\delta^{13}C$ values.

It turned out that the combination of $\delta^{13}C$ and $\delta^{15}N$ values from bulk organic shell matrix are not just excellent biosignatures for the discrimination of symbiotic or non-symbiotic lifestyle, but also allow distinguishing between thiotrophic and phototrophic symbiosis. For this, it is important to compare not the absolute values of C and N depletion between different species but rather values of species from same sampling site (see Fig. 1)!

This approach requires a sufficient quantity of preserved organic shell matrix. The insufficient conservation of the matrix during advanced diagenesis in fossil shells is the limiting factor for determination of biosignatures.

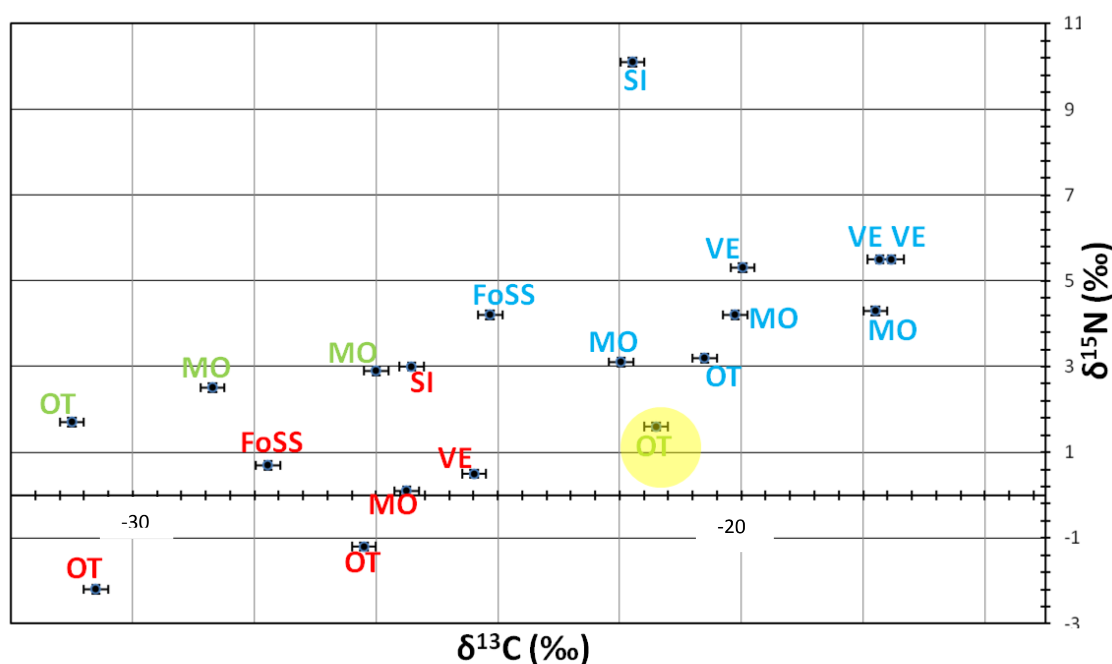

Fig. 1. A simplified diagram for comparison of all measured $\delta^{13}C$ and $\delta^{15}N$ values in shell organic matrix from different bivalve species in present study. Different lifestyles are color coded: (red) thiotrophic, (green) phototrophic, (blue) filter-feeding; Different sampling sites (OT) One Tree Island, (MO) Moreton Bay, (SI) Sicily, (VE) Venice, (FoSS) subfossil shells (see Chapter 3). Yellow circle: values from *Tridacna* shell with exceptional position (see Chapter 4).

As shown in chapter 4, microorganisms drill into the shells and possibly degrade the organic matrix in some cases. The comparison of a recent and a fossil *Tridacna* shell in chapter 4 clearly shows loss of matrix quality and quantity of the fossil shell. Thus, it was impossible to determine the isotopic composition of the fossil shell in this case.
Remarkably, the quality and quantity of the organic matrix of the subfossil shells from *Loripes* and *Venerupis* which were analyzed in chapter 3 appears to be identical to that of their recent shells.
These subfossil shells were sampled from a sediment core; thus they stay within the sediment under low oxygen conditions, which might be the reason for excellent matrix conservation of the shell after death of the bivalve. In contrast, the *Tridacna* shell, likewise from the Pleistocene, originated from an onshore outcrop and the amount of organic matrix was not sufficient to analyze its isotopic composition.
Both cases highlight the importance of environmental conditions for conservation of shell-organics during diagenesis. Thus, most promising for preservation of suitable isotopic biomarkers is a fast sedimentation and long period of time under oxygen-poor conditions.

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6. Summary

This thesis is focused on symbiosis between bivalves and autotrophic microorganisms (thiotrophic bacteria and phototrophic algae). The symbiotic relationship leads to special isotopic traces in the organics of the bivalves' shell. This thesis connects multiple isotopic values ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) of the bulk organic shell matrix and ($\delta^{13}C$) of shell-lipids with the respective pathways of carbon fixation, nitrogen assimilation and sulfur oxidation in thiotrophic bacteria. These characteristic isotopic patterns were compared to the isotopic composition of the shell-organics from non-symbiotic bivalves inhabiting the same habitat. In case of shells from living bivalves of the same sampling site, it was found that all isotopic values (including $\delta^{13}C$ values of shell-lipids) are lower in the organics of thiotrophic compared to non-symbiotic bivalves. Phototrophic bivalves have lower $\delta^{13}C$ values of bulk organic shell matrix compared to non-symbiotic bivalves as well. Phototrophic symbiosis could be differentiated from thiotrophic symbiosis, exhibiting lower $\delta^{15}N$ values.

It turned out that in the case of empty shells the composition and $\delta^{13}C$ values of shell-lipids and the $\delta^{34}S$ values of the bulk organic shell matrix are unstable and isotopic difference between bivalves' lifestyles remains inconsistent. Furthermore in one empty shell a contamination with boring microbes was observed. However, generally the quality of the bulk organic matrix was comparable to that of living shells and the isotopic difference of $\delta^{13}C$ and $\delta^{15}N$ values (bulk organic shell matrix) between symbiotic *vs.* non-symbiotic bivalve lifestyle was still stable in empty shells.

During further diagenetic processes the organic shell matrix could get lost. Under optimal conservation condition like in the case of the subfossil shells from the late Pleistocene (Adriatic sediments) isotopic composition in the remaining organic shell matrix could be identified. The results show that it was possible by using $\delta^{13}C$ and $\delta^{15}N$ values to deduce a non-symbiotic or thiotrophic lifestyle even for ancient bivalves.

Hence, $\delta^{13}C$ and $\delta^{15}N$ values of organic shell matrices could be a helpful tool to reconstruct evolution of symbiosis in bivalves and also other shelled mollusks.

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8. Curriculum vitae

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9.08-27.08.04	"Forschungsinstitut für die Biologie Landwirtschaftlicher Nutztiere", Dummerstorf, Group: Genetik u. Biometrie
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Since 1.06.10	PhD Student, Thesis: "Chemosynthetic Endosymbioses at recent and fossil Cold Seep-habitats".
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