

# Following the traces of symbiont bearing molluscs during earth history

Anne Dreier<sup>1,2</sup> \* & Michael Hoppert<sup>1,2</sup>

<sup>1</sup>*Institute of Microbiology and Genetics, Georg-August University Göttingen, Grisebachstr.8, 37077 Göttingen, Germany;*  
Email: [anne.dreier@geo.uni-goettingen.de](mailto:anne.dreier@geo.uni-goettingen.de)

<sup>2</sup>*Courant Centre Geobiology, Geoscience Centre, Georg-August University Göttingen, Goldschmidtstr. 3, 37077 Göttingen, Germany*

\* corresponding author

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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 molluscs and sulphur- 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 molluscs 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 analyses 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 where 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 molluscs. 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 molluscs.

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## 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 mol-

luscus, 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 as-

similation 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 molluscs 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 sulphur-oxidiser or methanotrophs belonging to the Gammaproteobacteria. According to phylogenetic analyses these symbioses have been established multiple times in earth history and evolved independently (Dubilier et al. 2008).

Several marine molluscs, 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 occur in the same microenvironments (Zhang & Millero 1993). The molluscs are able to bridge the oxic–anoxic boundaries using behavioral, morphological or metabolic adaptations and supply substrates (e.g., reduced sulphur 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 molluscs. 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 molluscs 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 mollusc 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 a mass occurrence of lucinid clams (Heterodonta: Lucinoida).

## Endosymbiotic molluscs

### Recent situation

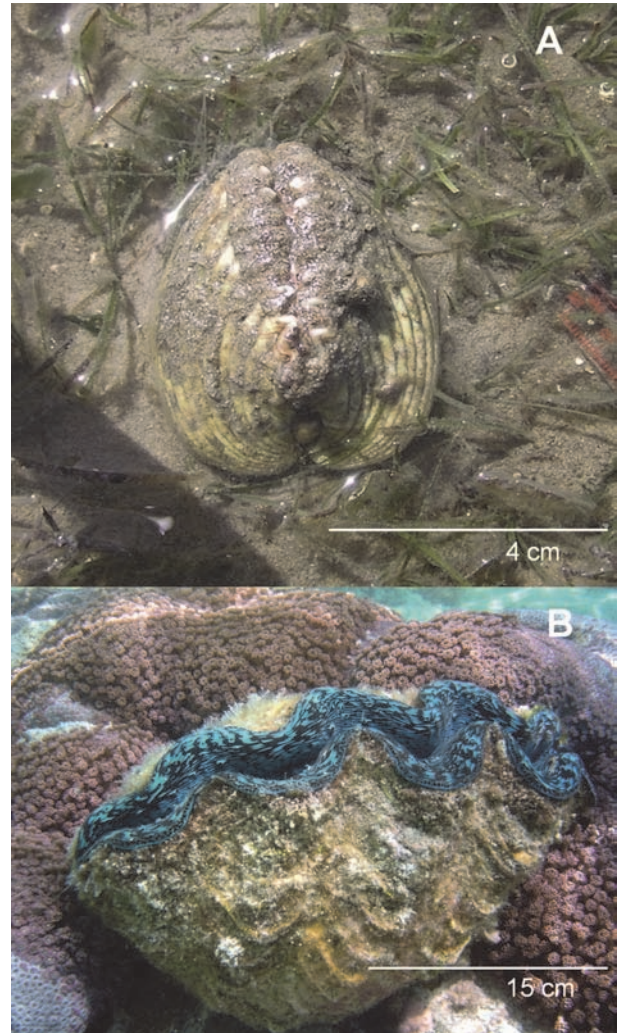
Within the clades of recent molluscs, endosymbiosis with sulphur- or methane-oxidising (chemosynthetic) bacteria occur in seven bivalve families: Solemyidae, Nucinellidae, Montacutidae, Mytilidae, Thyasiridae, Lucinidae (being the most diverse family), Vesicomidae (Taylor & Glover 2009, 2010; Taviani 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, Vesicomidae 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 molluscs of deep-sea habits are all three families of chemosynthetic gastropods, bathymodiolian mussels, vesicomid 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 molluscs 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 molluscs 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 (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 (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 Trapezüidae and Cardiidea (Fig. 2) maintain symbiotic associations with *Symbiodinium* (Yonge 1936; Kawaguti 1950, 1968, 1983; Purchon 1955; Stasek 1961; Hartman & Pratt 1976; 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* (Heterodonta: Veneroidea: Cardiidae) – mud flat of North Stradbroke Island, Queensland, Australia, and **(B)** *Tridacna maxima* (Heterodonta: Veneroidea: Cardiidae) – coral reef of One Tree Island, Queensland, Australia.

### *Symbiont-bearing invertebrates in earth's history*

Ancient shelled molluscs 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/molluscs 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 molluscs 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 analyses 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 & Tyler 2010), Lucinidae first appear during the 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). Vesicomysids 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 molluscs 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; Gill et al. 2005; Majima et al. 2005; Campbell 2006; Kiel & Little 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; Morton 2000; Coan et al. 2000; Schneider & Carter 2001). Recent members of Cardiidea with photosynthetic lifestyle 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 sulphur-rich conditions at the sea floor. Reduced oral structures and extended pleural areas were interpreted as an indication for a symbiotic relationship with sulphur 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 molluscs are relatively well-documented, not much attention is given to the non-seep related shallow water chemo- or phototrophic molluscs. At seep and vent sites the epifaunal molluscs densely colonise the habitat. Detecting fossil endosymbiosis in shallow water molluscs by using biogeochemical techniques has an advantage that possibly different heterotrophic molluscs 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 definitive non-symbiotic molluscs among the putative symbiotic ancestor of bathymodiolids is much greater 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).

## 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 molluscs 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 *Calypptogena* use their foot to dig for sulfide in the sediment and then use 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 molluscs lead to identifiable features or even “patterns”, identifiable in the fossil record?

In case that the major nutrients (carbon, nitrogen and sulphur) of molluscs derive from their prokaryotic symbionts, the host biomass is based on molecules built by the prokaryotic metabolism. In contrast, heterotrophic molluscs filter out or graze off particulate organic matter from their surrounding environment. Thus, search for fingerprints specific for either heterotrophic or chemo- and phototrophic molluscs must consider autotrophic and/or nitrogen metabolism of the symbionts.

### Carbon fixation

Apart from the cellulose-degraders in Teredinidae and methanotrophs, all prokaryotic symbionts fix inorganic carbon autotrophically. The common pathway for CO<sub>2</sub> 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 <sup>12</sup>CO<sub>2</sub> slightly faster than fixation of <sup>13</sup>CO<sub>2</sub> (Purich & Allison 2000). This selection leads to an enrichment of <sup>12</sup>C in the biomass relative to <sup>13</sup>C (negative δ<sup>13</sup>C value). The host obtains the organic carbon from its symbionts (Fiala-Médioni & Felbeck 1990; Childress & Fisher 1992), hence δ<sup>13</sup>C ratios of host tissue reflect the carbon source. Mentionable is the fact that <sup>13</sup>C depletion by chemoautotrophic bacteria using the Calvin-Benson cycle for CO<sub>2</sub> 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 velum* and *Bathymodiolus thermophilus*, exhibiting relatively low δ<sup>13</sup>C ratios, whereas form II RubisCO is expressed in the tubeworms *Riftia pachyptila* and *Tevnia jerichonana* with higher δ<sup>13</sup>C ratios (Childress & Fisher 1992). However, the δ<sup>13</sup>C ratios are also influenced by the ratios of source carbon (CO<sub>2</sub>) 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 δ<sup>13</sup>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 <sup>12</sup>CH<sub>4</sub> which leads to a further de-

pletion in δ<sup>13</sup>C values (Coleman et al. 1981; Grossman et al. 2002).

In summary, δ<sup>13</sup>C values of tissue from molluscs which harbor, chemoautotrophs and/or methanotrophs (“primary producers”) are all significantly depleted relative to molluscs at higher trophic levels. This depletion pattern should also be expected for tissue of phototrophic molluscs

### Nitrogen assimilation

The main nitrogen sources of bacterial biomass and hence host tissue in chemoautotrophic symbioses are ammonia (NH<sub>4</sub><sup>+</sup>) 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). Molluscs 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 <sup>14</sup>NH<sub>3</sub> (Lee & Childress 1994). Independent of the pathway of nitrogen assimilation, it is known that δ<sup>15</sup>N ratio increases by about 3.4 ‰ per trophic level (Minagawa & Wada 1984; Peterson & Fry 1987). Accordingly, primary producers must show lower δ<sup>15</sup>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).

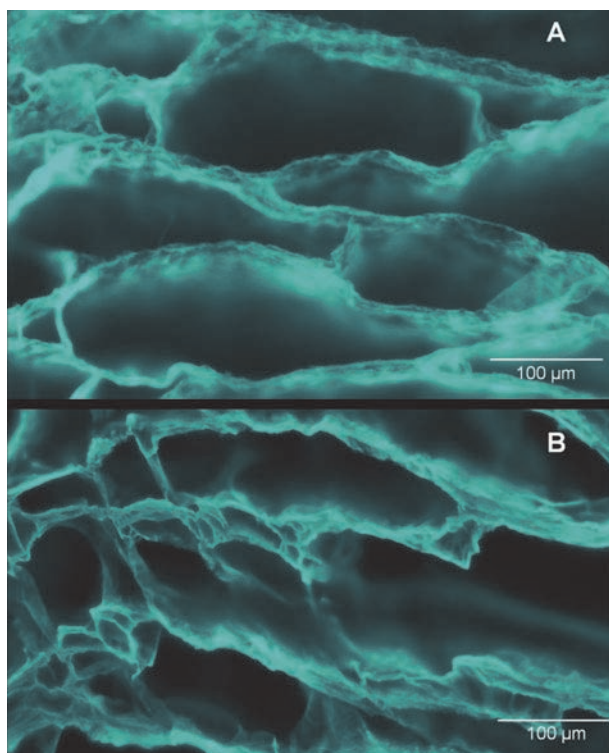
### Sulphur oxidation

Thiotrophic endosymbiosis is most common among molluscs (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 δ<sup>34</sup>S (Kaplan et al. 1963; Kiyosu & Krouse 1993; Aharon & Fu 2000; Joye et al. 2004). The pathway of sulphur oxidation does not lead to a significant fractionation of sulphur 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 sulphur compounds of non-thiotrophic molluscs derive from sea-water sulfates with δ<sup>34</sup>S ratios being markedly different from that of sulfides in sediments (Kaplan et al. 1963; Trust & Fry 1992; Michener & Schell 1994).

**Table 1:** Some  $\delta^{13}\text{C}$  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).

$\delta^{13}\text{C}$	Organisms	Carbon assimilation	$^{12}\text{C}$ enrichment
-30 ‰ to -34 ‰	Bivalves with chemoautotrophic symbionts	$\text{CO}_2$ to organic C (RubisCO form I)	22–30 ‰, (24.4 ‰, form IA)
-8.8 ‰ to -16 ‰	Hydrothermal vent vestimentiferan tubeworms with chemoautotrophic symbionts	$\text{CO}_2$ to organic C (RubisCO form II)	18–23 ‰
-18 ‰ to -28 ‰	Phytoplankton	$\text{CO}_2$ to organic C (supposed RubisCO form IB)	22–30.3 ‰
-39.3 ‰ (thermogenic $\text{CH}_4$ ) to -76.0 ‰ (biogenic $\text{CH}_4$ )	Bivalves with methanotrophic symbionts (in some cases additional chemoautotrophic symbionts)	$\text{CH}_4$ to organic C	5–30 ‰

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



**Fig. 3:** Chitin staining of a cross section of *Tridacna maxima* decalcified shell. (A) Recent, One Tree Island, Queensland, Australia; (B) Pleistocene, north of Dahab, Sinai, Egypt. Cross sections were stained with Calcofluor White.

In summary, the isotopic compositions of the biological elements carbon, sulphur 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 molluscs, preserved biomolecules are needed. Here one may take benefit from the mineralised mollusc shells, which are perfect long term conservation wrappings for organic matter.

### Different biosignatures and stability over geological timescales

Various techniques are used to detect symbiotic prokaryotes in mollusc tissue, such as 16S ribosomal DNA sequence analysis, fluorescent *in situ* hybridisation and transmission electron microscopy.

After death, soft tissue is degraded and only shells are left for incorporation into the fossil record. Mollusc 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 ( $\beta$ -sheet), and the presence of chitin (Weiner & Traub 1980; Weiner et al. 1983).

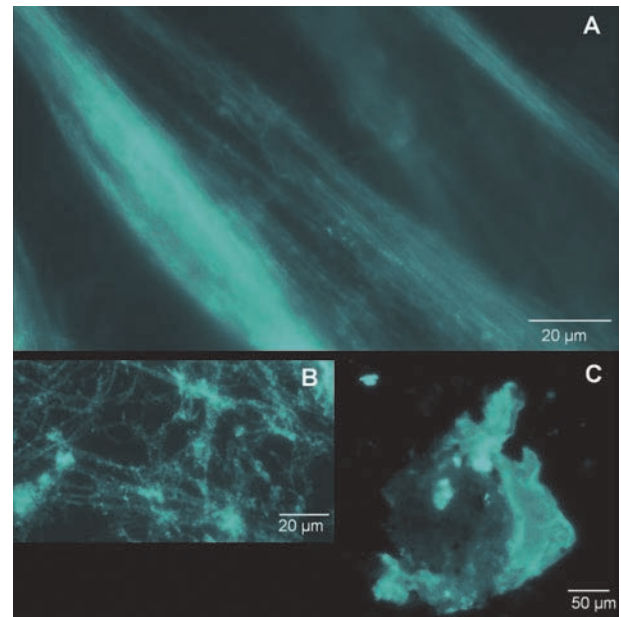
Recent studies about shell proteins imply that the organic shell matrix is composed of a macromolecular framework consisting of a chitin-silk fibronin gel with acidic proteins (e.g., Marin & Luquet 2007; Evens 2008; Marin et al. 2008). To demonstrate the presence of chitin in shells, staining with the fluorescence dye Calcofluor White may be performed, which binds to cellulose and chitin. Obviously, also the remaining organic shell matrix of fossil shells could be stained. This could be a hint for the persistence of these biomolecules in the shell matrix. Fig. 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). It is obvious that the Calcofluor-stained material in the fossil shell of *Tridacna maxima* (Fig. 4C) is different from the filamentous structure in the modern shell (Figs. 4A, 4C).

In addition, a cross section of a fossil (Upper Cretaceous) *Inoceramus* sp. shell was stained with Calcofluor White. Treatment of cross section was identical to that of *Tridacna* but without decalcification. Fig. 5 shows that maybe fossil chitin was stained between the calcium carbonate crystals of the *Inoceramus* sp. shell.

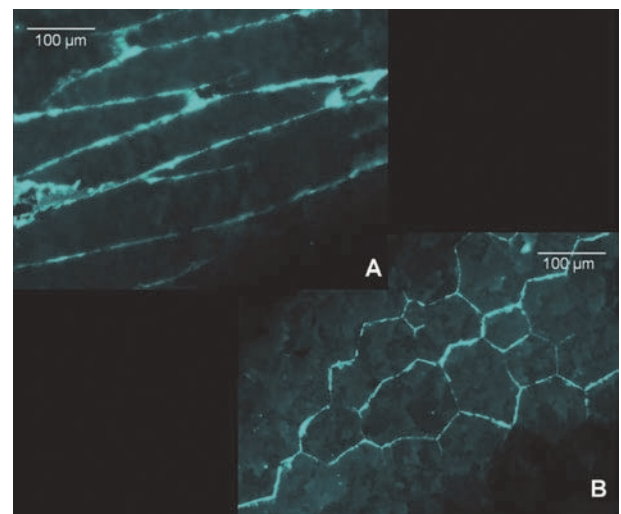
The preserved biomolecules of the shell will provide information about the mollusc's diet. In endosymbiont-bearing molluscs, carbon, nitrogen and sulphur are taken up by the symbionts, get an isotopic fingerprint and are then incorporated in mollusc biopolymers (see above). Since the remains of the organic 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 molluscs. In many studies  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and sometimes  $\delta^{34}\text{S}$  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; Lorrain et al. 2002; Dattagupta et al. 2004; 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  $\delta^{34}\text{S}$  values of the organic matrix of empty shells from recent bivalves and subfossil (Late Pleistocene) shells.

It was shown that sulphur isotopes are not useful markers to detect ancient the thiotrophic lifestyle, because after death of the molluscs  $\delta^{34}\text{S}$  values in the organic matrix will decrease. It was assumed that the reason could be the instability of sulphur-containing amino acids (Jones & Vallyntyne 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  $\delta^{34}\text{S}$  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  $\delta^{34}\text{S}$  ratio dropped slightly from 7.8 ‰ to 7.5 ‰. Longer resting time in the sediment is needed to further decrease the  $\delta^{34}\text{S}$  ratio further (Dreier et al. 2012).



**Fig. 4:** Chitin staining of decalcified piece of *Tridacna maxima* shell. (A–B) Recent, One Tree Island, Queensland, 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.



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

The enrichment of sulfides during degradation of soft tissue may be the cause of the framboidal pyrite formation (Fig. 5; Berner 1984; Wilkin 1995) and for the decreasing  $\delta^{34}\text{S}$  ratio of shells after death.

Lipids are another prominent group of biochemical markers, which are analyzed to identify symbiosis in molluscs. 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; Fullerton et al. 1995). In molluscs the lipid content depends on dietary lipid intake (Moreno et al. 1980; Piretti et al. 1987), thus lipid content of molluscs 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  $\delta^{13}\text{C}$ , 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,  $\delta^{13}\text{C}$  ratios of most molluscs 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 chemotrophic bivalves to be more depleted in  $\delta^{13}\text{C}$  relative to heterotrophic bivalve. 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,  $\delta^{13}\text{C}$  values of fossil shell lipids have been not reported so far.

## 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).

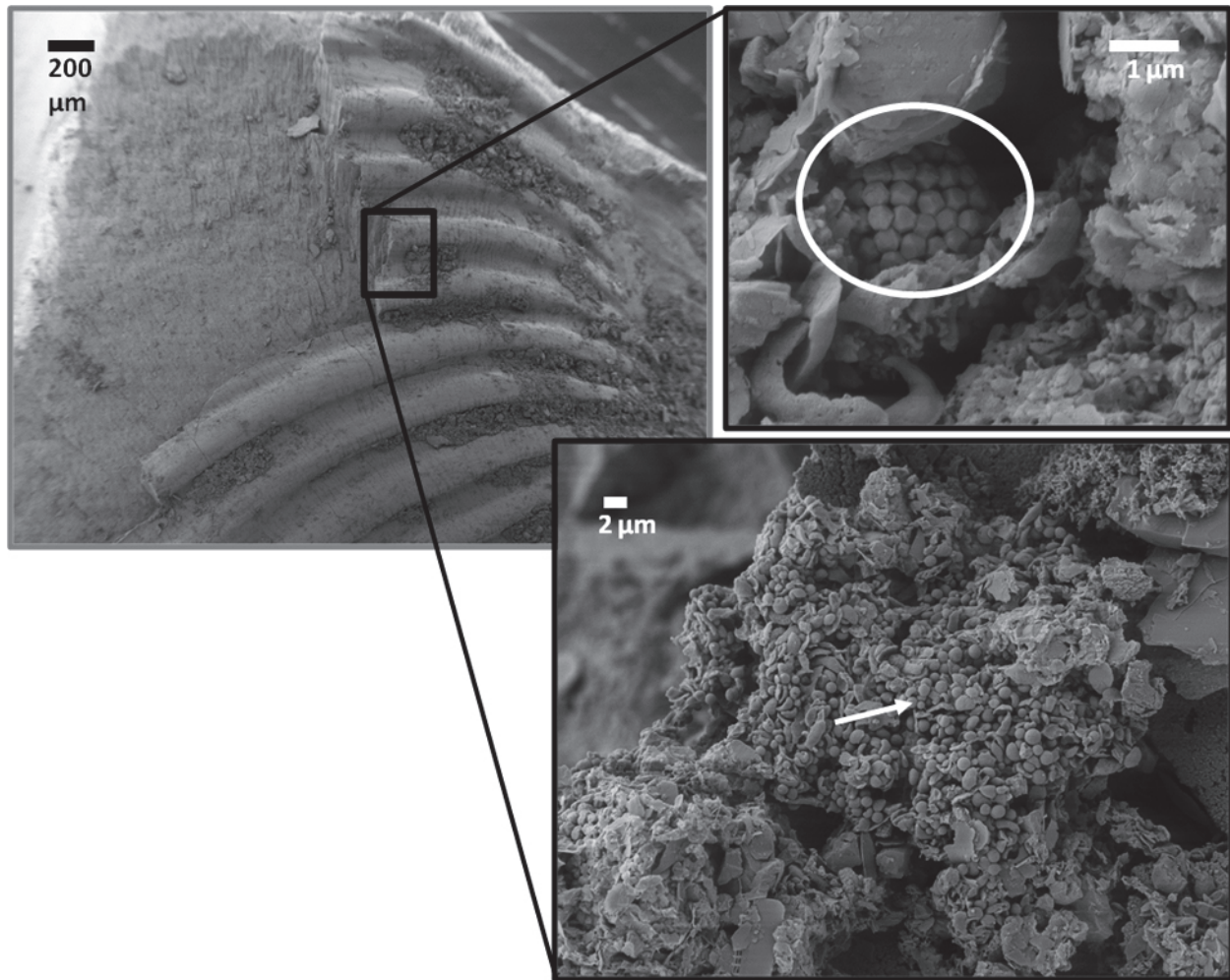
If we even could identify the point where the lifestyle of a species switches from heterotrophic to symbiotic, we will be also able to find factors driving emergence of cooperative microbial-host associations, which will foster our understanding of this evolutionary driving force. Molluscs are very suitable 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 molluscs shells, it is possible to distinguish between “primary consumers” (chemo- and phototrophic) and molluscs 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  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values for the heterotrophic bivalve *Venerupis* ( $\delta^{13}\text{C}$  of -24.1 ‰ and  $\delta^{15}\text{N}$  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 molluscs are not completely based on their symbionts. Some of the molluscs still use filter-feeding as an additional option (Duplessis et al. 2004). To date no isotopic data are available for chemo-compared with phototrophic molluscs inhabiting the same site, so is not known if there is a resolution limit between the two different primary producer's 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 molluscs – 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.





**Fig. 6:** Scanning electron micrographs of a *Venerupis* shell (Heterodonta: Veneroidea: Veneridae) 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].

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