Fluid venting structures of terrestrial mud volcanoes (Italy) and marine cold seeps (Black Sea) -Organo-geochemical and biological approaches

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Überall geht ein früheres Ahnen

dem späteren Wissen voraus.

Alexander von Humboldt

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Chapter 1: Introduction

1.1 Fluid venting structures

Fluid venting is an important mechanism for transferring water, gas (in particular methane), higher hydrocarbons and sometimes sediments from the lithosphere to the hydrosphere and atmosphere. Such focused fluid flows occur in terrestrial and marine environments worldwide and are closely related to tectonically active zones (active and passive continental margins; Fig. 1).

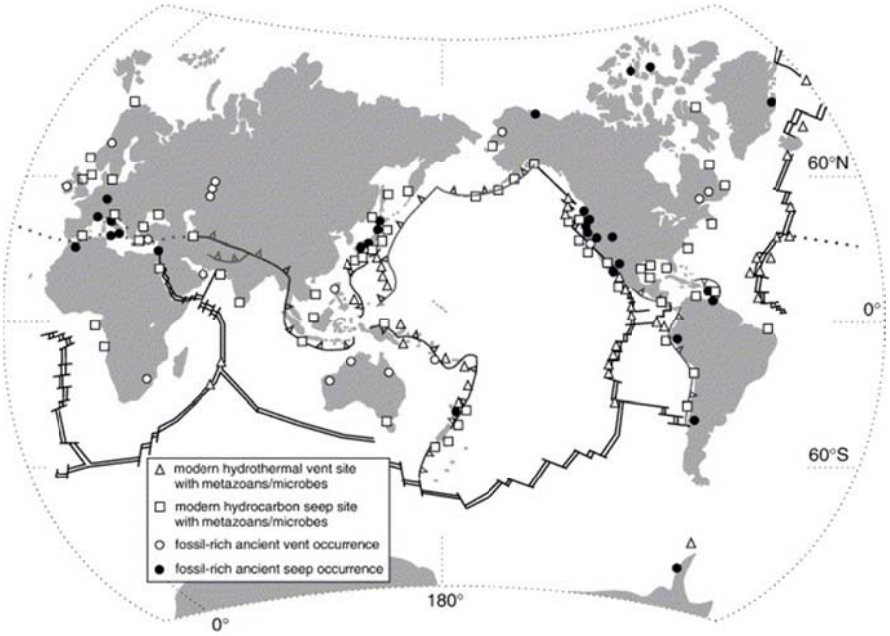

Figure 1: Worldwide distribution of ancient and modern fluid venting structures – hydrothermal vents and cold seeps along tectonic active zones (Campbell et al. 2006) with kind permission of Elsevier.

Due to tectonic stress (e.g. subduction or diapirism) faults are formed, which induce the formation of physically weak pathways, where the fluids may arise (Brown, 1990; Milkov, 2000; Kopf, 2002; Kopf et al., 2001; Dimitrov, 2002; Niemann and Boetius, 2010). The migration of the fluids to the surface leads to the formation of characteristic structures, which can reach diameters of a few millimeters up to several hundreds of meter (Dimitrov, 2002). On the seafloor, the emissions lead to the formation of pockmarks (Hovland and Judd, 1988), large fissure eruptions of sediments from the subsurface (Ivanov et al., 1998), mud volcanoes (Dimitrov, 2002) and microbially formed carbonate build ups, a unique characteristic in the anoxic water body of the Black Sea (Ivanov et al., 1991; Luth

et al., 1999; Peckmann et al., 2001; Lein et al., 2002; Michaelis et al., 2002; Reitner et al, 2005 a, b). Onshore, mud volcanoes and more abundant micro-seepage occur, due to methane venting (Etiope et al., 2009). Density differences, gas advection driven by pressure gradients, compression of the pore water by increasing pressure in the sediments and permeability (Darcy´s Law) along weak pathways in the sediments are the main driving forces for the upward rising of the fluids (Brown 2000; Etiope and Martinelli, 2002; Niemann and Boetius, 2010). Pore fluids, for examples, contain methane, light hydrocarbons or other reduced compounds which decrease the density of the pore fluids causing their migration to the surface through the permeable sediments or along fault, fractures, bedding planes and other pathways (Brown, 2000; Etiope and Martinelli, 2002). Another reason for the upward migration of not only pore but also mud fluids is an increasing pressure arising from compaction within the sediments due to a high sedimentation rate, burial of sediments by slope failures or tectonic stress (Brown 1990; Dimitrov, 2002; Kopf et al., 2001, 2002; Mellors et al., 2007; Milkov, 2000) resulting in the formation of methane driven mud volcanoes. The slow gas diffusion driven by concentration gradients (Fick´s law) is only important for long-term and small scale gas flow in more homogeneous porous media e.g. as primary migration of hydrocarbons from source rocks to reservoirs, and plays only a role in marine environments and cannot be invoked for terrestrial seeps (Etiope et al., 2009a).

1.2 Methane emissions

As mentioned above methane is the main component of the expelled fluids of the different fluid venting structures and is, due to the absorption of long wave radiation emitted from the surface, a strong greenhouse gas and contributes to the global warming (Lacis et al., 1981; Hansen et al., 1988; Ramanathan, 1988). Beside the man-made sources (e.g. biomass burning, rice fields, waste treatment, ruminants and landfills), which make up 62 % of the methane emission, the main source of natural methane emissions are wetlands, termites and aquatic systems which account for 30 % of the total methane emission (Etiope et al., 2004). Nevertheless, 7 % of the global methane emissions are due to natural geological sources, including mud volcanoes, micro-seepage, geothermal fluxes and marine seepage (Etiope et al., 2004). The greenhouse effect of methane in general, is

more than 20 times higher than that of carbon dioxide (Kvenvolden, 1988; Forster et al., 2007). Thus, the understanding of the geological sources is important for the understanding of the global climate system and the climate change. Methane can be formed through different processes. Most important are biogenic processes, where methane is produced by microorganism due to degradation of organic matter and carbon dioxide reduction, and less important thermogenic processes, where it is formed during thermal degradation of organic compounds.

1.2.1 Biogenic methane

Methanogenesis, the process which causes the formation of biogenic methane, is the final step of the anaerobic degradation of organic matter, where methane is formed as by-product (Thauer, 1998). In anaerobic freshwater systems, for example, most of the organic matter (glucose from cellulose) is converted to carbon dioxide and methane (Thauer, 1998). Carbon dioxide, hydrogen, formate, methanol, methylamines and acetate are the main substrates which are linked to this process and, therefore, to the generation of methane (Thauer, 1998). Most important among them are hydrogen and acetate, which give rise to two different kinds of methanogenesis: (i) the fermentation of acetate (eq. 1,2,3,4 and 5) and (ii) carbonate reduction (eq. 6), which can be represented by the general reactions (Thauer, 1998; Whiticar, 1999).

(i) Fermentation to acetate (Thauer, 1998; Whiticar, 1999):

$C_6H_{12}O_6 + 2\,H_2O \rightarrow 2\,CH_3COO^- + 2H^+ + 2\,CO_2 + 4\,H_2$ [eq. 1]

$C_6H_{12}O_6 + 2\,H_2O \rightarrow 2\,CH_3COO^- + 2\,HCOO^- + 4\,H^+ + 2\,H_2$ [eq. 2]

and finally the conversion to methane:

$CH_3COO^- + H^+ \rightarrow CO_2 + \mathbf{CH_4}$ [eq. 3]

$4\,HCOO^- + 4\,H^+ \rightarrow 3\,CO_2 + \mathbf{CH_4} + 2\,H_2O$ [eq. 4]

$4\,H_2 + CO_2 + H^+ \rightarrow \mathbf{CH_4} + 2\,H_2O$ [eq. 5]

(ii) Carbonate reduction (Thauer, 1998; Whiticar, 1999):

$CO_2 + 8H^+ + 8e^- \rightarrow \mathbf{CH_4} + 2\,H_2O$ [eq. 6]

A third metabolic pathway is called methyl-group reduction (Thauer, 1998).

These processes are restricted to methanogenic archaea, a phylogenetic diverse group of strictly anaerobic euryarchaeota (Garcia et al., 2000). The stable carbon isotopic composition of methane ($\delta^{13}C\text{-}CH_4$) produced by carbonate reduction are in the range from -80 to -60 ‰ (vs. VPDB), while methane produced by fermentation process ranged from -60 to -50 ‰ (vs. VVPDB; Whiticar, 1999). During the microbial formation of methane only small amounts of other light hydrocarbons were produced. In comparison to the thermogenic generation of methane, the microbial methane formation is carried out at a relatively shallow depth where appropriate thermodynamic conditions i.e. relative low temperatures and pressures, prevail. Increasing temperatures and pressure in the sediments inhibits the activity of the methanogens.

1.2.2 Thermogenic methane

Thermogenic gas is formed in the deep subsurface, where conditions are appropriate (high pressure, >120°C), and therefore, remaining organic matter in the sediments could be degraded into gas and oil (Kotelnikova, 2002). With increasing depth of the source sediments, temperatures and pressure rise, and the thermogenic methane generation start. Furthermore, other light hydrocarbons, e.g. ethane, propane and butane were produced. The stable carbon isotopic composition of methane ($\delta^{13}C\text{-}CH_4$) produced by thermal cracking of sedimentary organic matter to higher hydrocarbons and gas are in the range from -50 to -20 ‰ (vs. VPDB; Whiticar, 1999).

1.2.3 Abiogenic methane

Furthermore, small amounts of abiogenic methane and higher hydrocarbons can be formed during (i) cooling and degassing of mafic igneous rocks and (ii) by serpentinization of ultramafic rocks (Charlou et al., 1998). During the latter process, olivine and pyroxene, minerals contained in the mantle, were oxidized in the presence of water. Molecular hydrogen is formed, and Fe(II) bonded in minerals is oxidized to Fe(III), which leads to the formation of magnetite. Following a type of reaction called “Fischer-Tropsch”, the hydrogen reacts with carbon dioxide, which is either released from mantle minerals or is part of the seawater, to form methane and subsequently higher hydrocarbons. Magnetite and/or elements

like nickel, cobalt and other, which are part of the mantle minerals, act as catalysator (Szatmari, 1989; Coveney et al., 1987; Reeburgh, 2007).

$$6\,[(Mg_{1.6}\,Fe_{0.4})SiO_4] + 7\,H_2O \rightarrow 3\,[(Mg,Fe,Ni)_3Si_2O_5(OH)_4] + Fe(II)Fe(III)_2O_4 + \mathbf{H_2} \quad \text{[eq. 7]}$$

(olivine) (serpentine) (magnetite)

$$CO_2 + 4\,\mathbf{H_2} \rightarrow \mathbf{CH_4} + 2\,H_2O \quad \text{[eq. 8]}$$

This process describes the dominant methane source in hydrothermal vent systems especially along mid-ocean ridges and above serpentine diapirs (Charlou et al., 1998). The stable carbon isotopic composition of methane ($\delta^{13}C\text{-}CH_4$) produced by abiogenic processes ranges from -53 to -19 ‰ (vs. VPDB), and may overlap the $\delta^{13}C\text{-}CH_4$-values that are typical for biogenic methane (Horita and Berndt, 1999).

1.2.4 Secondary microbial methane

A further source of methane gas is the subsurface anaerobic biodegradation of oil or natural gas. Nitrogen, methane, and carbon dioxide are the dominant gases produced during this process (Mikov et al., 2011). It is suggested that methanogenic biodegradation in subsurface oil and gas reservoirs most likely occurs through the oxidation of higher hydrocarbons to acetate and hydrogen (eq. 9), linked to syntrophic acetate oxidation (eq. 10) and methanogenesis from carbon dioxide reduction (eq. 11; Dolfing et al., 2008; Jones et al., 2008; Milkov et al., 2011):

$$4\,C_{16}H_{34} + 64\,H_2O \rightarrow 32\,CH_3COO^- + 32\,H^+ + 68\,H_2 \quad \text{[eq. 9]}$$

$$32\,CH_3COO^- + 32\,H^+ + 64\,H_2O \rightarrow 64\,CO_2 + 128H_2 \quad \text{[eq. 10]}$$

$$196\,H_2 + 49\,CO_2 \rightarrow 49\,\mathbf{CH_4} + 98\,H_2O \quad \text{[eq. 11]}$$

Usually, the $\delta^{13}C\text{-}CH_4$- values of secondary microbial methane ranged from the -35 ‰ to -25‰ (Roberts and Aharon, 1994). Furthermore, anaerobic biodegradation processes are characterized by large isotopic separations between successive n-alkanes and high C_2H_6/C_3H_8 ratio (C_2/C_3) (Pallasser, 2000; Waseda and Iwano, 2008). In particular, the secondary methanogenesis has a direct effect on the $\delta^{13}C$-values of the carbon dioxide. The $\delta^{13}C\text{-}CO_2$ of the second microbial methane formation exceeds values of +10‰. Therefore, a positive $\delta^{13}C\text{-}CO_2$ value

determined in emitted gases from fluid venting structures indicates biodegradation processes and the formation of secondary methane gas in the subsurface petroleum reservoirs.

1.2.5 Stable carbon and hydrogen isotopes and the identification of gas *o*rigin

Generally, the chemical composition (methane, ethane, propane, and butane) and the stable carbon and hydrogen isotopes are the basis to determine the origin of the emitted gases from cold seeps and mud volcanoes (Etiope et al., 2009). In particular, the source of the methane gas could be identified by plotting the carbon isotopic data versus the hydrogen isotopic values (Fig.2; $\delta^{13}C\text{-}CH_4$ vs. $\delta D\text{-}CH_4$; Whiticar, 1999) or by plotting the carbon isotopic values versus the light hydrocarbon composition of the gas ($\delta^{13}C\text{-}CH_4$ vs. $CH_4/ (C_2H_6 + C_3H_8)$; Bernard et al., 1978). For example, extremely negative carbon isotopic values ($\delta^{13}C–CH_4 < -50‰$ (vs. VPDB)) with a high ratio of the light hydrocarbon composition in the gas ($C_1/(C_2 + C_3)$) indicates a biogenic origin of the methane gas. Nevertheless, it has to be noted that the application of these cross-plots can provide misleading information due to the fact that post-genetic alteration processes can affect the isotopic composition of the emitted gas (Etiope et al., 2009b).

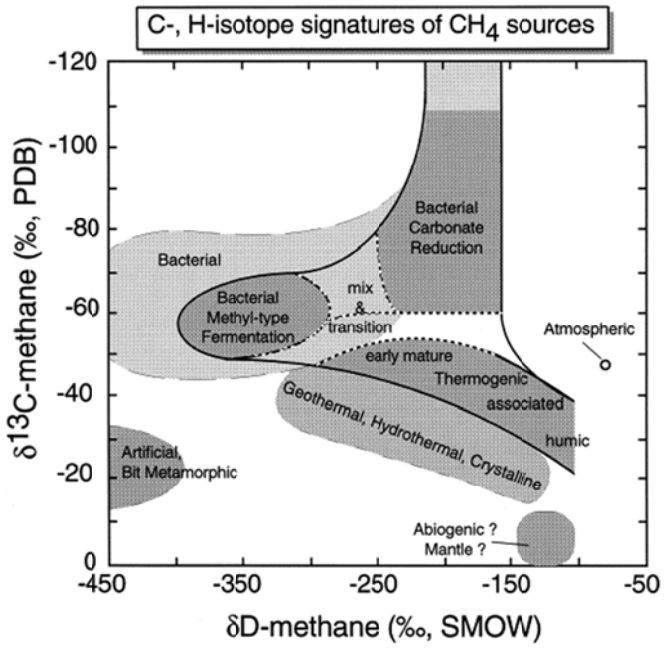

Figure 2: Classification of bacterial and thermogenic gas shown in a $\delta^{13}C\text{-}CH_4$ vs. $\delta D\text{-}CH_4$-diagram (Whiticar, 1999) with kind permission of Elsevier. Results for the mud volcanoes are shown in chapter 4.

Possible post-genetic processes that are possible are: (a) aerobic and anaerobic oxidation of methane; (b) abiogenic oxidation; (c) isotopic fractionation by diffusion; (d) molecular fractionation by advection; (e) gas mixing and (f) anaerobic biodegradation of petroleum and secondary methanogenesis. But is has to be noted that not all of them or none of them affects the cold seep and mud volcano systems (Etiope et al. 2009b). Abiogenic oxidation processes, for example, occur where temperatures reach 80 °C to 400 °C, which is not very common in mud volcano systems where temperatures are usually below 80°C (Etiope et al., 2009b).

1.3 Cold seeps - involved processes

1.3.1 Anaerobic oxidation of methane (AOM)

1.3.1.1 Microorganisms

The anaerobic oxidation of methane is one of the post-genetic alteration processes which can affect systems of the different fluid venting structures that occur worldwide. Furthermore, most of the produced methane in marine sediments is consumed by the anaerobic oxidation of methane (Knittel and Boetius, 2009). Therefore, the AOM is an important process, which controls the methane emissions from the ocean into the atmosphere (<2 % of the global flux; Judd et al., 2002; Reeburgh, 2007). The AOM is extensively studied and documented for the marine methane venting structures (Knittel and Boetius, 2009 and references therein).

Geochemical observations in the 1970s and 1980s provide the first evidence for the removal of methane within anoxic sediments and seawaters (Reeburgh, 1969, 1976; Barnes and Goldberg, 1976; Martens and Berner, 1977). Until now, it is known, that the anaerobic oxidation of methane (AOM) is mediated by a consortia of methanotrophic archaea and sulfate reducing bacteria (SRB) (Hinrichs et al., 1999; Boetius et al., 2000; Orphan et al., 2001a; Michaelis et al., 2002; Knittel et al., 2003, 2005; Niemann et al., 2006a, b). The previously known groups of anaerobic methanotrophs (ANME-1, -2, -3) are distantly related to methanogens of the orders *Methanosarcinales* and *Methanomicrobiales* (Boetius et al., 2000; Orphan et al., 2001b, 2002; Knittel et al., 2005; Niemann et al., 2006a; Lösekann et al., 2007). ANME-2 groups (Fig. 3a), distantly related to *Methanosarcinales* and

the ANME-1 group (Fig. 3b), which is distantly related to the *Methanomicrobiales*, forms a consortium together with sulfate-reducing bacteria (SRB) of the *Desulfosarcina/ Desulfococcus* (DSS) group bacteria (Pimenov et al., 1997; Michaelis et al., 2002; Tourova et al., 2002; Blumenberg et al., 2004). SRB, related to the *Desulfobulbus* group (Niemann et al., 2006; Lösekann et al., 2007) are the syntrophic partner of the ANME-3 group, which is also distantly related to *Methanosarcinales* (Orphan et al., 2001b; Niemann et al., 2006).

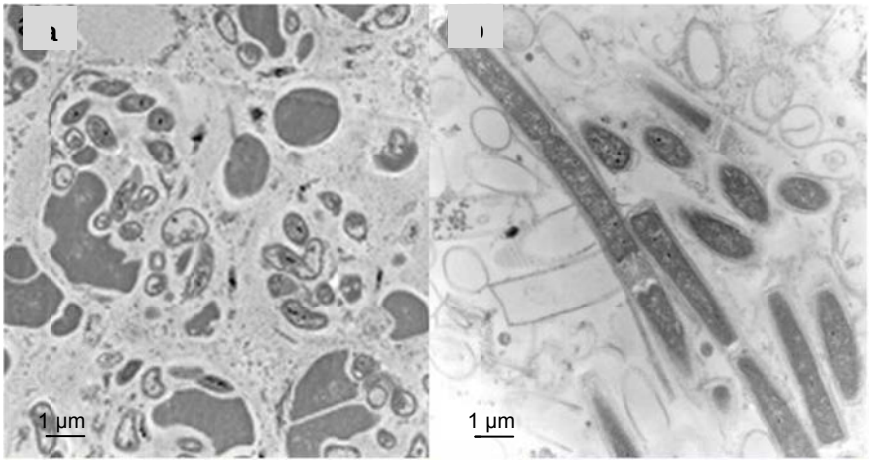

Figure 3: Consortia of archaea and sulfate reducing bacteria (SRB) (a) Transmission electron microscopic (TEM) micrograph of ANME-2/greigite-bearing SRB; (b) TEM micrograph of the cylindric-shaped ANME-1. (Pictures are from Reitner et al., 2005).

Recently, a fourth group has been found and was described as ANME-2d or GoM Arc I. The clade was named GoM Arc I, because it is not monophyletic with the other ANME-2 subgroups (Mills et al., 2005; Lloyd et al., 2006; Martinez et al., 2006) and until now, it was not proven whether it mediates the AOM or whether it forms a consortium with sulfate reducing bacteria (Knittel and Boetius, 2009).

1.3.1.2 Diagnostic lipid biomarkers

In addition to molecular techniques (e.g. 16S rDNA clone libraries, fluorescence in situ hybridization (FISH)) lipid biomarker analyses provide a way to identify the AOM communities. Eukaryotes, bacteria and archaea are characterized by partially specific lipid biomarkers, which are components of the cytoplasmic membrane surrounding each cell (Fig. 4). Moreover, ratios of stable carbon isotopes in the specific biomarkers contain information on the biosynthesis of lipids, the carbon fixation pathways (Boschker et al., 1998; Alberts et al., 2002;

Madigan et al., 2002; Brocks and Pearson, 2005) and especially on the carbon substrate used (Hayes, 2001).

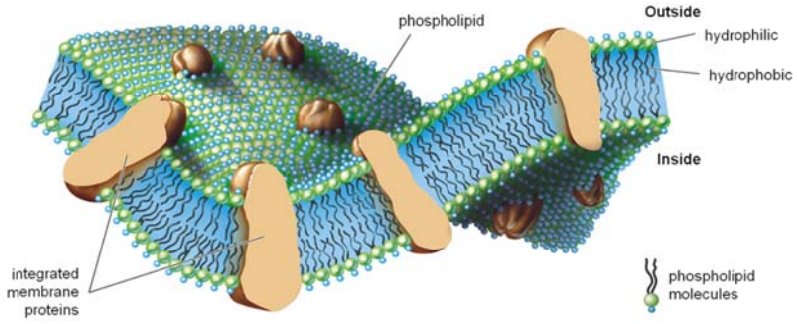

Figure 4: Schematic illustration of cytoplasmic membrane consisting of phospholipids with hydrophilic and hydrophobic parts and proteins (according to Madigan et al., 2006).

An excellent example is the AOM, where the usually isotopically light methane carbon is transferred into the lipids of the closely operating AOM performing consortia of sulfate-reducing bacteria and methane oxidizing archaea (e.g., Hinrichs et al., 1999; Pancost et al., 2001b; Blumenberg et al., 2004). Thus, the carbon isotope signatures of the specific AOM lipids should be extremely depleted in ^{13}C (<50‰ vs. VPDB). Therefore, the lipid biomarker signatures in combination with its specific carbon isotope ratios can be used to trace AOM communities in modern and ancient environments (Thiel et al., 1999; Peckmann and Thiel, 2004). Typically, the presence of the ANME-archaea is evidenced by the lipid biomarker signatures of the isoprenoidal glycerol ethers (archaeol and *sn*-2-hydroxyarchaeol), the C_{20} and C_{25}, irregular tail-to-tail linked, isoprenoidal hydrocarbons 2,6,11,15-tetramethyl-hexadecane (crocetane) and 2,6,10,15,19-pentamethyleicosane (PMI) and their unsaturated homologues (Kushwaha and Kates, 1978; Koga et al., 1993, 1998; Bian, 1994; Bian et al., 2001; Elvert et al., 2001; Blumenberg et al., 2004; Koga and Mori, 2005; Niemann et al., 2006).

But, archaeol for example could be found in a variety of archaea and is therefore not specific for the AOM. Thus, the occurrence of a few of these biomarker signatures is only indicative for AOM if the isotope signatures of the specific biomarkers are depleted in ^{13}C. The same is true for the associated sulfate-reducing bacteria (SRB). None of the bacterial lipid signatures is exclusively

restricted to one specific group (Hinrichs and Boetius, 2002) and only a strong depletion in ^{13}C is indicative for a contribution to the AOM (Hinrichs et al., 2000; Orphan et al., 2001a; Michaelis et al., 2002; Zhang et al., 2002; Elvert et al., 2003; Blumenberg et al., 2004).

Fatty acid glycerol esters (commonly cleaved and separately analyzed as fatty acid methyl esters – FAMEs) and in particular the terminally branched $C_{15:0}$, $C_{16:1\omega5}$, cy-$C_{17:0\omega5,6}$, and $C_{17:1\omega6}$ are used to identify the AOM-associated sulfate reducers (Niemann et al., 2008). The *iso*- and *anteiso*-branched $C_{15:0}$ fatty acids have been found in most of the AOM environments worldwide (Niemann et al., 2008). Furthermore, some non-isoprenoidal dialkyl glycerol diethers (DAGEs) are used for the fingerprinting of the sulfate reducing bacteria (Pancost et al., 2001; Elvert et al., 2005; Stadnitskaia et al., 2005). Thiel et al. (1999) and Hinrichs et al. (1999) have given the first evidence for anaerobic methanotrophy based on the isolation of archaeal lipids depleted in ^{13}C.

1.3.1.3 Metabolic process

Recent phylogenetic and biochemical studies have suggested that the ANME-archaea have supposedly reversed the methanogenic pathway (Fig. 5). Current models suggest that methane is converted by methanotrophic archaea to carbon dioxide and reduced by-products, which are subsequently consumed by sulfate-reducing bacteria (Hoehler et al., 1994). These by-products, which are transferred to the syntrophic partners, are still unknown. As intermediates formate (Sörensen et al., 2001), hydrogen, acetate and methanol have been suggested, but all of these compounds were excluded by in vitro feeding studies (Nauhaus et al., 2002; Widdel et al., 2006). Methyl sulfides were also suggested (Moran et al., 2008), but could also not be proved (Knittel and Boetius, 2009). A further hypothesis proposed a transfer of reducing equivalents via electron shuttles. Tests with compounds like phenazines and humic acids gave also no positive results (Nauhaus et al., 2005).

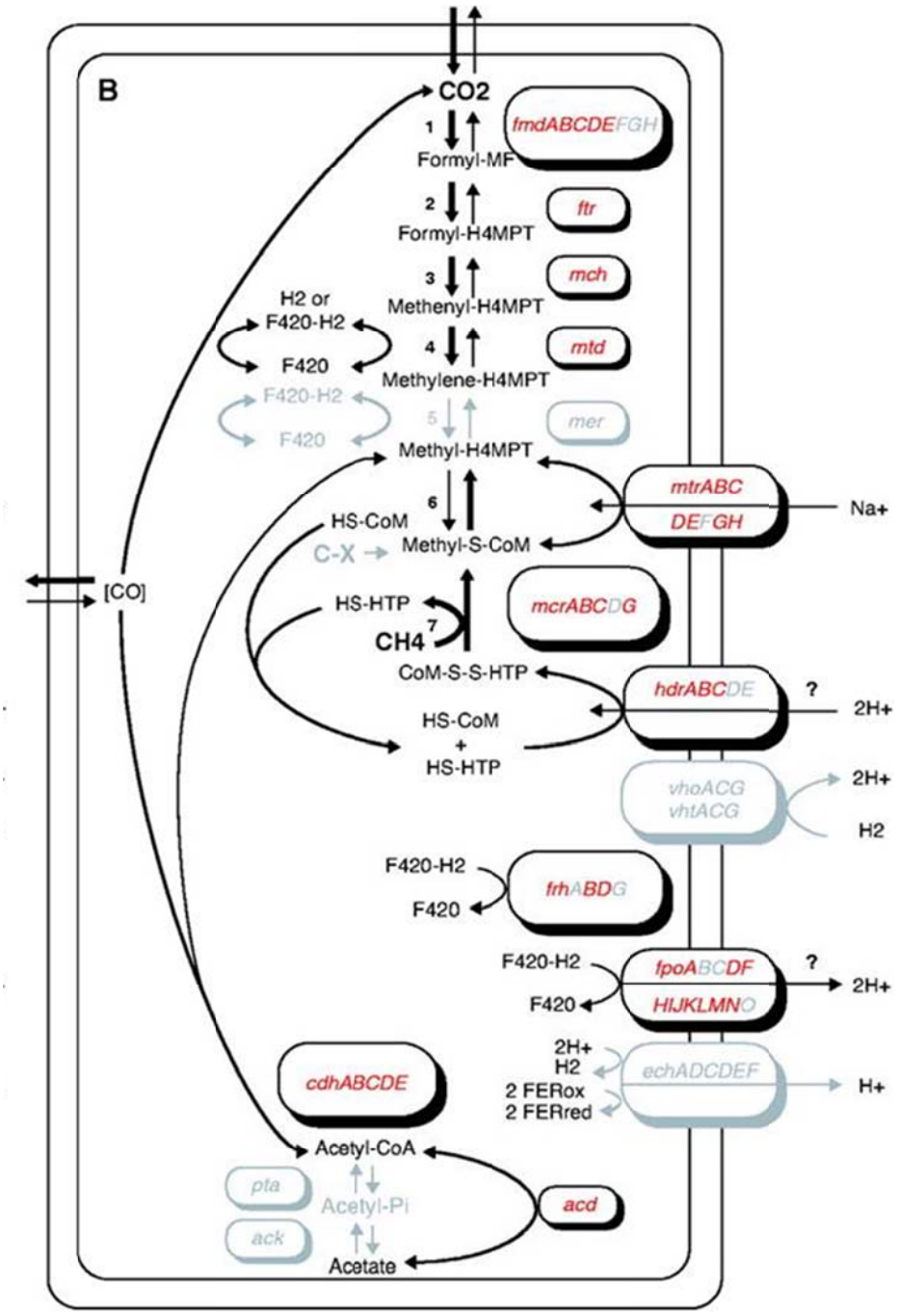

Figure 5: A pathway of the reconstructed reverse Methanogenesis for ANME-1 based solely on predicted gene content of identified ANME-1 fosmids. Positive gene identifications are shown in red. Negative gene identifications are shown in gray "from [Hallam et al., 2004, Science; 305:1457--1462]. Reprinted with permission from AAAS."

Nevertheless, most of the genes associated with the methanogenesis were identified in ANME-1- and ANME-2-groups (Fig. 5; Hallam et al., 2003, 2004). Additionally, Krüger et al. (2003) found a conspicuous Ni-protein in the microbial reef structures, which is similar to the terminal enzyme of archaeal methanogenesis (methyl coenzyme M reductase, MCR) and most likely involved in the AOM. MCR catalyzes the final methane forming step of methanogenesis in

archaea (see chapter 2). The reaction takes place under strictly anoxic conditions. To date, most of the evidences for the reversed methanogenesis hypothesis are based on the analysis of the biofilm genomes (Hallam et al., 2003, 2004) or purification of the enzyme obtained from a protein extract of the whole microbial mat (Krüger et al., 2003). Recently, the adenosine-5'-phosphosulfate /ammonium peroxodisulfate (APS) reductase, an enzyme specific for the sulfate reduction pathway, was detected on cellular and sub-cellular level (Wrede, 2011). Furthermore, a novel mechanism based on nitrate, was discovered for the AOM (Eq. 12 and Eq. 13). An enrichment culture from a water drain in the Netherlands was grown in a methane atmosphere with nitrate and nitrite as electron acceptors (Raghoebarsing et al., 2006; Ettwig et al., 2008, 2010):

$$5\ CH_4 + 8\ NO_3^- + 8\ H^+ \rightarrow 5\ CO_2 + 4\ N_2 + 14\ H_2O \qquad \text{[eq. 12]}$$

$$3\ CH_4 + 8\ NO_2^- + 8\ H^+ \rightarrow 3\ CO_2 + 4\ N_2 + 10\ H_2O \qquad \text{[eq. 13]}$$

1.3.2 Aerobic oxidation of methane

1.3.2.1 Microorganisms

Aerobic methanotrophy is conducted by groups of methanotrophic bacteria, instead of methanotrophic archaea and sulfate reducing bacteria described for anoxic conditions (AOM). The methanotrophic bacteria, a subgroup of the methyltrophs, are strictly aerob and obligate methylotroph, which means that they can use only one carbon compounds (CH_4, CH_4O) or carbon compounds without carbon bonds as carbon source. They can be divided into two phylogentic groups; Type I methanotrophs belonging to the γ-Proteobacteria and Type II methanotrophs belonging to the α-proteobacteria (Hanson and Hanson, 1996; Madigan et al., 2006; Bowman, 2006). The genera *Methylosphaera*, *Methylobacter*, *Methylomicrobium*, *Methylomonas*, *Methylococcus* and *Methylocaldum* were described for the Type I methanotrophs. Due to phylogenetically and morphologically distinctions to the other Type I methanotrophs, *Methylococcus* and *Methylocaldum* are often referred as "type X methanotrophs" (Bowman, 2006; McDonald et al., 2008). The genera *Methylosinus, Methylocystis*, *Methylocella* and *Methylocapsa* were assigned to the Type II methanotrophs (McDonald et al., 2008). The methane oxidation was

performed at intracytoplasmic membranes, which are characteristic for both types of methanotrophs (Hanson and Hanson, 1996). Nevertheless, the intracytoplasmic membranes of the two groups were arranged in different parts of the cells. These methanotrophic bacteria are able to utilize single-carbon compounds (Bowman, 2006). Methane acts as energy source (electron donor) as well as the partial carbon source for methanotrophs (Hanson and Hanson, 1996). To mediate the aerobic oxidation of methane, Type I methanotrophs used the ribulose monophosphate pathway, whereas Type II methanotrophic bacteria used the serine pathway.

1.3.2.2 Diagnostic lipid biomarkers

The presence of aerobic methanotrophic bacteria is typically evidenced by the occurrence of specific hopanoids, in particular 3-methylhopanoids, 4-methylated steroids and some fatty acids. Diagnostic steroids are 4,4-dimethyl and 4a-methyl sterols and for the hopanoids, 3-methyl-17β(H)21β(H)-*bis*homohopanacids, diploptene, diplopterol, 3β-methyl diplopterol (Bird et al., 1971; Bouvier et al., 1976; Zundel and Rohmer, 1985a). But it has to be noted that diplopterol, diploptene, and their diagenetic derivatives should only be used with caution, because anaerobic as well as aerobic bacteria were identified as alternative source organisms (Pancost et al., 2000; Sinninghe Damsté et al., 2004; Härtner et al., 2005; Blumenberg et al., 2006; Birgel et al., 2008).

For this reason, 3-methyl hopanoids and intact polyhydroxylated pentacyclic triterpenoids, so called bacteriohopanepolyols (BHPs), specifically those with a NH_2 group at C_{35} (35-amino-BHPs), were used as specific biomarker for aerobic methanotrophic bacteria (e.g. Neunlist and Rohmer, 1985a; Neunlist and Rohmer, 1985b; Zundel und Rohmer, 1985; Cvejic et al., 2000; Talbot et al., 2001; Blumenberg et al., 2006, 2007, 2009). 35-aminobacteriohopane-30,31,32,33,34-pentol (aminopentol), for examples, have been found only in Type I methane-oxidizing bacteria (Neunlist and Rohmer, 1985; Zhou et al., 1991; Cvejic et al., 2000a), whereas 35-aminobacteriohopane-31,32,33,34-tetrol (aminotetrol) is less specific, because it is produced by all types of methanotrophs, as well as some sulfate reducing bacteria (Blumenberg et al., 2006, 2009).

Further diagnostic signatures are specific phospholipid ester-linked fatty acids. C_{14} and C_{16} phospholipid ester-linked fatty acids are specific for Type I methanotrophic bacteria, whereas C_{18} is abundant in Type II methanotrophic bacteria (Bodelier et al. 2009). In addition, Type I methanotrophic bacteria contain $C_{16:1\omega8c}$ and $C_{16:1\omega5t}$ fatty acids, Type II contain, in contrast, $C_{18:1\omega8c}$ fatty acids (Nichols et al., 1985; Bowman et al., 1991).

1.3.2.3 Metabolic process

During the aerobic oxidation of methane or methanotrophy, methane is oxidized with molecular oxygen to methanol, formaldehyde, formate, and finally to carbon dioxide (Hanson and Hanson, 1996, Madigan et al., 2006, Bowman, 2006). Specific enzymes are needed to carry out the process of aerobic methanotrophy. The first step of this process, the oxidation of methane with molecular oxygen to methanol and water, is catalyzed by an enzyme called methane monooxygenase (MMO). There a two types of this enzyme: (1) the copper-containing particulate methane monooxygenase (pMMO) and (2) iron-containing soluble methane monooxygenase (sMMO) (Hanson and Hanson, 1996). The first one is abundant in all methanotrophs, whereas the latter is only contained in some strains (Hanson and Hanson, 1996). The second step of this process is mediated by an enzyme called methanol dehydrogenase (MDH), which converts methanol to formaldehyde. It is the key enzyme for methanotrophic as well as methylotrophic bacteria (Hanson and Hanson, 1996; Lidstrom, 2006). Expressed MDH was detected in the anoxic environment of the Black Sea by Wrede (2011), which indicates that both the anaerobic oxidation of methane, as dominant process, as well as the aerobic methane oxidation takes place in the Black Sea cold seeps and the associated anoxic water body (Wrede, 2011).

1.3.3 Methanogenesis

1.3.3.1 Microorganisms

As mentioned above, methanogenesis is the final step of the degradation of organic matter and is restricted to strictly anaerobic methanogenic archaea, a phylogenetical diverse group of euryarchaeota (Garcia et al., 2000). They are classified in the five orders *Methanobacteriales*, *Methanococcales*, *Methano-microbiales*, *Methanopyrales* and *Methanosarcinales* (Thauer, 1998).

1.3.3.2 Diagnostic lipid biomarkers

Due to the fact, that it is postulated that the anaerobic methane-oxidizing archaea (ANME) reversed the methanogenic pathway diagnostic lipid biomarker signatures, for example archaeol and hydroxyarchaeol, are the same for both metabolic processes. Therefore, PMI (2,610,15,19-pentamethylicosane) is diagnostic for methanogenic archaea and/or anaerobic methane oxidizing archaea, whereas crocetane (2,6,11,15-tetramethylhexadecane) was not found in methanogenic archaea. Only the strong depletion of ^{13}C which can only developed during the oxidation of methane, in all of the mentioned biomarker signatures, makes it possible to distinguish between both processes.

1.3.3.3 Metabolic process

There are three possible methanogenic pathways: (1) the fermentation of acetate, (2) carbonate reduction and (3) the methyl-group reduction (Thauer, 1998). These processes were described in detail in chapter 1.2.1.

1.4 Research Areas

Figure 6 shows the location of the studied fluid methane seeps. The cold seep carbonate towers of the Black Sea and the mud volcanoes in Northern Italy and Sicily. The first study site is a marine methane seep, whereas the latter are terrestrial mud volcanoes. Both areas are characterized by active fluid venting.

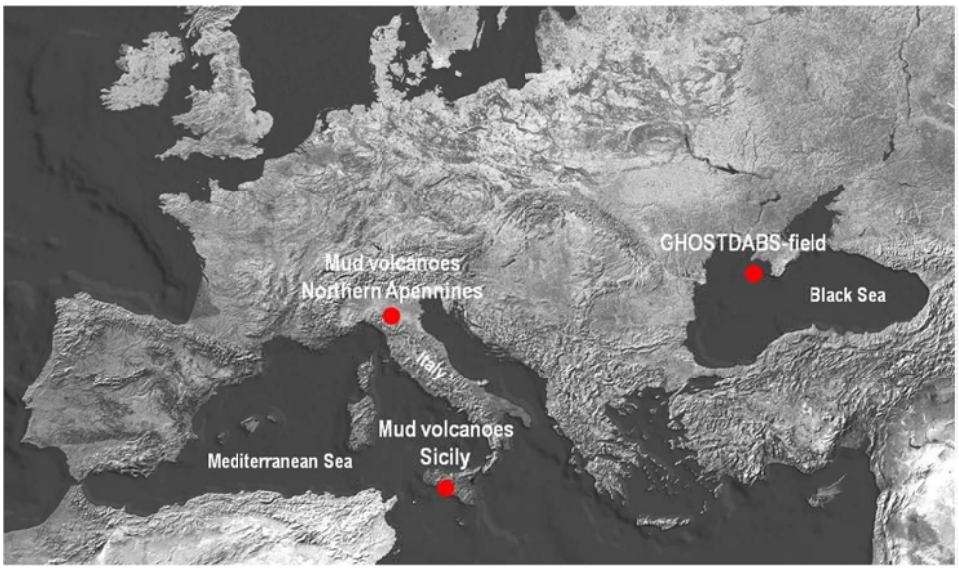

Figure 6: Map of Europe (http://www.dlr.de) with sample locations in Northern Italy, Sicily and the Black Sea.

1.4.1 Black Sea cold seeps

Cold seep systems can be regarded as chemosynthesis-based ecosystems that support large amounts of life in deep seas (Schubert, 2011). Sites with intense fluid venting occur in several areas of the Black Sea (Polikarpov et al., 1997; Stadnitskaia et al., 2005 and references therein), the samples used in this study were collected at the lower Crimean shelf (N44° 46.510' N, E31° 59.570') at approximately 230 m water depth in the permanent anoxic water body of the Black Sea (Luth et al., 1999; Michaelis et al., 2002), explored during a German–Russian–Ukrainian joint expedition (GHOSTDABS) in 2001. Due to the strict stratification of the water body, the Black Sea is a unique ecosystem and cold seep carbonate build ups (Fig. 7), which can protrude several meters into the anoxic water column, can be formed.

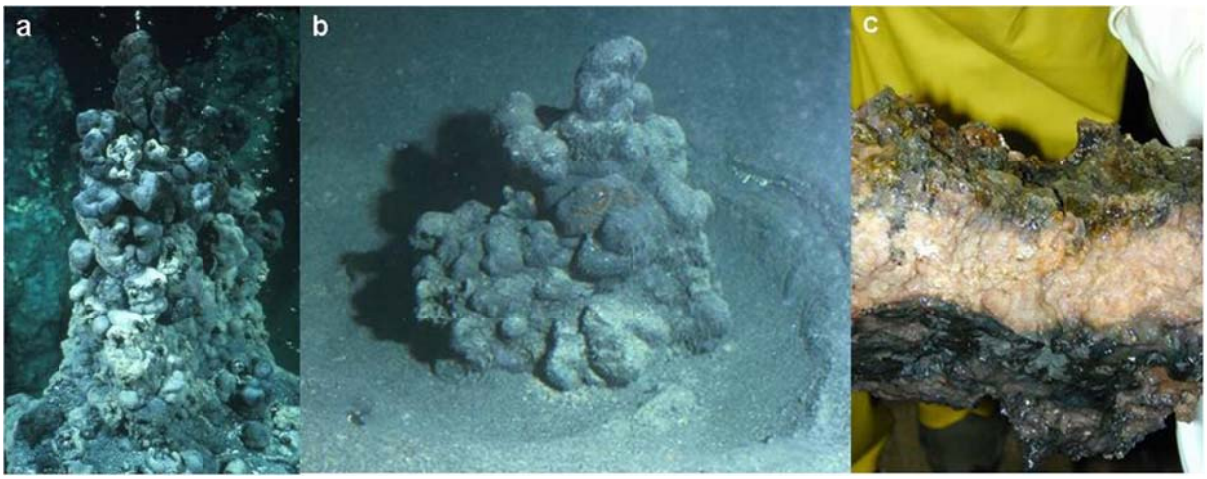

Figure 7: (a) and (b) Methane-derived carbonate structures of the Black Sea and (C) associated microbial mats. The black layer in direct contact with the anoxic seawater; the orange-colored layer in the middle and the greenish layer in the innermost part of the structures. (Photo: GHOSTDABS - BMBF & Jago team: K. Hissmann & J. Schauer)

The stratification of the Black Sea is caused by a difference in salinity between the deep and the surface water which was established due to the inflow of high salinity waters of the Mediterranean Sea invading the Black Sea via the Bosporus after the last glaciation. Therefore, a permanent stratification was established between the original freshwater body with low salinity and the underlying marine water body with a higher salt concentration (Peckmann et al., 2001). This distinct stratification and the emission of the methane-rich fluids out of the sediment result in an extensive activity of anaerobic oxidation of methane. This process increases the carbonate alkalinity according to the overall reaction (cf. Eq. 14; Reitner et al., 2005b) and, therefore, the formation of distinct carbonate deposits (Goedert and Squires, 1990; Ivanov et al., 1991; Luth et al., 1999; Peckmann et al., 1999; Greinert et al., 2001; Aloisi et al., 2002; Campbell et al., 2002; Lein et al., 2002; Michaelis et al., 2002; Peckmann and Thiel, 2004; Reitner et al., 2005a; Reitner et al., 2005b; Pape et al., 2008):

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \qquad \text{[eg. 14]}$$

These carbonate deposits can be divided into two groups: (1) formed beneath the sediment-water-boundary layer as plates of carbonate-cemented sediments, crusts and large tabular constructions (Reitner et al., 2005a, b) and (2) below the chemocline in the permanent anoxic water body as tall carbonate build-ups and tower-like structures which can reach several meters in height (Fig. 7 a, b; Peckmann et al., 2001; Lein et al., 2002; Michaelis et al., 2002). A close-up view

on these cold seep carbonate structures of the Black Sea revealed a distinct internal structure. The carbonate deposits were formed by several distinct layers of microbial mats (Fig. 7c), which can reach several centimeters in diameter. The microbial mats that are typically associated with the methane derived carbonates of the Black Sea can be divided into three different layers (Michaelis et al., 2002). The outer surface of the microbial reef is covered by a black layer, which is in direct contact to the permanently anoxic water of the Black Sea. This few millimeter thick layer consists of consortia of archaea which are affiliated to the *Methanosarcinales* (ANME-2 group) and sulfate-reducing bacteria (SRB) of the *Desulfosarcina/ Desulfococcus* group bacteria (Blumenberg et al., 2004). SRB of this mat type exhibit often intracytoplasmic magnetosome-like chains of greigite precipitations and droplets resembling storage inclusions of polyhydroxyalkanoates (PHA; Reitner et al., 2005a, b). The underlying orange-colored layer is mainly composed of the cylindrically shaped ANME-1 archaea (Michaelis et al., 2002) which are distantly related to Methanomicrobiales (Hinrichs et al., 1999) and embedded SRB colonies. The innermost part of the carbonate towers is formed by a greenish layer, which exhibit a considerably higher microbial diversity than the other two mat types (Reitner et al., 2005 a, b).

1.4.2 Terrestrial mud volcanoes in Italy

Although the anaerobic oxidation of methane has been mainly found in marine sediments and methane seeps, it has been recently found in a terrestrial mud volcano area near Paclele Micci in Romania (Alain et al., 2006). Mud volcanoes are another characteristic morphological feature of the diverse fluid venting structures. They can be found in marine and terrestrial environments worldwide and are formed by the emission of argillaceous material, water, brine, gas and oil (Milkov, 2000). They occur in active tectonic compression zones or areas with high sedimentation rates and are caused by various geological processes. An abnormally high pore fluid pressure and sediment instabilities caused by tectonic accretion and faulting, slope failures (olistostromes), high sedimentation rates or fluid emissions from mineral dehydration lead to the expulsion of fluids (gas, sediment and water) (Brown, 1990; Dimitrov, 2002; Kopf et al., 2001, 2002; Mellors et al., 2007; Milkov, 2000).

Terrestrial mud volcanoes in Italy occur mainly in the Northern Apennines, Central Italy and Sicily (Martinelli and Judd 2004). In the Northern Apennine chain, mud volcanoes occur along the external compressional margin, mostly located in the Emilia-Romagna region (Conti et al., 2003). A case in point is the Natural Reserve of “Salse di Nirano” near Modena (Fig. 8), one of the largest mud volcano areas in Italy (Martinelli and Judd, 2004). Here, the mud cones can reach 5 m in height and small mud polls which occur beside these main cones.

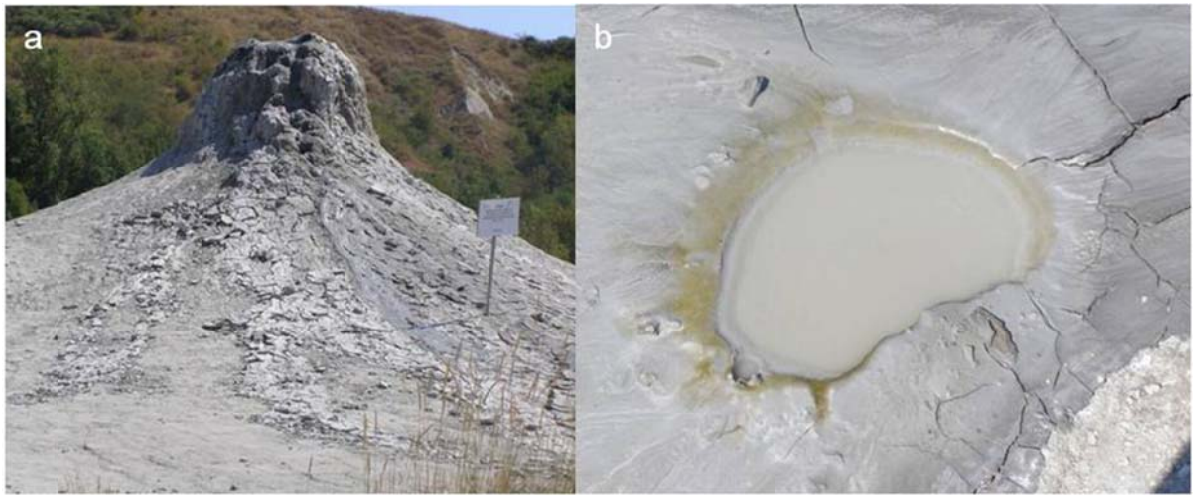

Figure 8: (a) Mud volcano cone and (b) a small mud pool of the Salse di Nirano mud volcano area located in the Northern Apennines.

This study is mainly focused at mud volcanoes located in the Northern Apennines (e.g. Salse di Nirano; N44° 30' 49.68" E10° 49' 24.6"). Moreover, further mud volcanoes in Northern Italy and Sicily, Italy were examined.

1.5 Main goals of the work

The anaerobic oxidation of methane (AOM) is a major sink of this greenhouse gas in marine systems (Reeburgh, 1996; Hinrichs and Boetius, 2002; Knittel and Boetius, 2009 and references therein). Up to 85% of the methane from deeper sediment horizons is oxidized by AOM in the upper anoxic parts of sediments (Ehalt, 1974; Iversen, 1996). Key participants in the Black Sea cold seeps are ANME-1 (anaerobic methane-oxidizing archaea)/DSS consortia (Michaelis et al., 2002; Knittel et al., 2005; Meyerdierks et al., 2005) and ANME-2/greigite-bearing DSS consortia (Reitner et al., 2005a, 2005b). The microbiology and physiology of these consortia have been investigated in several studies (Pimenov et al., 1997;

Michaelis et al., 2002), but little is known about the exact metabolic activities of the cells.

In **Chapter 2 "Immunological localization of Coenzyme M reductase in anaerobic methane-oxidizing archaea of ANME1 and ANME 2 type" (Heller et al., 2008, Geomicrobiology Journal) was described.** The Immunogold labeling technique was used to detect and determine the organisms that host Ni-containing MCR, the key enzyme of the (reverse) methanogenesis, in the microbial mats of the Black Sea.". MCR is located and expressed in both the ANME 1 and ANME 2 cell types.

Chapter 3 "Nickel signatures as a geochemical indicator for the anaerobic oxidation of methane in recent and ancient microbial mats" (Heller et al., in preparation) describes a geochemical indicator for the anaerobic oxidation of methane (AOM). Laser Ablation - Inductively Coupled Plasma - Mass Spectrometry (LA-ICP-MS) analyses of the microbial mats and different carbonate phases of the Black Sea cold seeps show that nickel revealed a distinct distribution pattern in the different samples. In this regard, two questions arise: (1) can fossil methane seeps reveal the same nickel distribution pattern and (2) can nickel be a geochemical indicator for the anaerobic oxidation of methane or for methanogenesis? To answer these questions, further LA-ICP-MS analyses were performed on recent Black Sea samples and on fossil seep samples from Montepetra, Italy.

In recent times, knowledge of marine AOM habitats has increased steadily, but there are only few data available about terrestrial methane venting structures. Alain et al. (2006) detected AOM in terrestrial mud volcanoes in Romania. Hence, to expand our knowledge, the terrestrial mud volcanoes of Northern Italy and Sicily were studied in detail in this thesis. The mud volcanoes of the Salse di Nirano located in the Northern Apennines were of particularly interest. This study includes geochemical and organo-chemical analyses of the released fluids to obtain a deeper insight into the system of the mud volcanoes and to determine the different geochemical and microbial processes that occur in these systems.

Chapter 4 "The expelled mud volcano fluids (gas, water and sediment particles): first attempt" (Heller et al., in prep) describes the molecular and

isotopic compositions of the emitted gas of the mud volcanoes in Northern Italy and Sicily. The results show that the released methane gas has a thermogenic origin, followed by biodegradation and secondary methanogenic processes in the associated petroleum reservoirs. The geochemistry of the water helps identify the source of the emitted fluids. Here, deep reservoirs (depths of 2 to 3 km) are the main source of the expelled fluids and are where AOM and sulfate reduction possibly occur.

The organo-geochemical analyses (lipid biomarker) were performed at the Salse di Nirano mud volcanoes. **Chapter 5 "Geomicrobiology of fluid venting structures at the Salse di Nirano mud volcano area in the Northern Apennines (Italy) (Heller et al. 2011a; Lecture Notes of Earth Science)** shows that sulfate-reducing bacteria and methanotrophic archaea were found in the fluids, which confirms that the AOM takes place in terrestrial mud volcanoes. Nevertheless, due to only slightly depleted stable isotope ratios of the specific biomarker, AOM plays only a minor role. The majority of the microorganisms are neither involved in AOM nor live on other ^{13}C-depleted carbon sources.

Furthermore, sediments that the fluids had passed through were extracted to differentiate between the autochthonous and allochthonous sources of the lipid biomarkers, as described in **Chapter 6 "Terrestrial mud volcanoes of the Salse di Nirano (Italy) as a window into deeply buried organic-rich shales of Plio-Pleistocene age" (Heller et al., 2011b, Sedimentary Geology)**. The results show that most of the organic matter in the fluids determined by lipid biomarker analyses had other origins than recent or sub-recent microbial processes.

In **Chapter 7**, which is a brief summary of both parts of this thesis, the cold seep structures of the Black Sea and the terrestrial mud volcanoes in Italy are were presented. The samples of the Black Sea cold seeps are eminently suitable for identifying the metabolic activities of the involved microorganisms, whereas the terrestrial mud volcanoes are windows into the deep biosphere and provide information about the microbial and geochemical processes taking place at these depths.

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Chapter 2

Immunological localization of coenzyme M reductase in anaerobic methane-oxidizing archaea of ANME 1 and ANME 2 type

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Abstract

The Black Sea is a large, euxinic marine basin, in which the anaerobic oxidation of methane (AOM) plays an important role in the carbon cycle. Methane-related carbonate build-ups, found on the NW´ Black Sea shelf are part of a unique microbial ecosystem. Two archaeal guilds are mainly responsible for the AOM: ANME-1 (anaerobic-methane-oxidizing communities)/DSS consortia and ANME-2/greigite-bearing DSS-consortia. These microorganisms constitute a significant sink of methane on earth, but despite their relevance for the global carbon cycle little is known about the biology of AOM. Phylogenetic and biochemical analyses suggested that ANME-archaea have supposedly reversed the methanogenic pathway. Here, we were able to localize methyl coenzyme M reductase (MCR), which catalyzes the final step of the methane formation, in ultrathin sections. The result was obtained by the immunogold labeling technique using a specific antiserum against the MCR. This technique revealed that the MCR is located in both ANME-1- and ANME-2-archaea. The data also show that MCR-like enzymes are not only encoded in the genomes of ANME-1 and ANME-2, but are, in fact, expressed as cellular proteins at high levels.

Keywords cold methane seeps, Black Sea, anaerobic oxidation of methane, sulfate reducing bacteria, methayl coenzyme M reductase, Immunogold labeling technique, ANME -1, ANME -2

2.1 Introduction

The microbially mediated anaerobic oxidation of methane (AOM) supposedly follows the overall chemical equation $CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$. AOM is now identified as a major sink of the greenhouse gas in marine systems (Reeburgh, 1996; Hinrichs and Boetius, 2002 and references therein). Up to 85% of the methane from deeper sediment horizons is oxidized by AOM in upper anoxic parts of the sediments (Ehalt, 1974; Iversen, 1996). Obviously, AOM is carried out by a symbiotic association of methanogenic archaea and sulfate reducing eubacteria, namely members of the *Methanosarcinales* and the *Desulfosarcina/Desulfococcus* group (DSS) (e.g., Hinrichs et al., 1999; Thiel et al., 1999; Boetius et al., 2000; Orphan et al., 2001a, b; 2002; Elvert et al., 2003; Reitner et al., 2005a, b; Treude et al., 2005). In the Black Sea the key participants are two archaeal guilds: ANME-1 (anaerobic methane-oxidizing archaea)/DSS consortia (Boetius et al., 2000; Hinrichs et al., 1999; Michaelis et al., 2002; Orphan et al., 2001b; Knittel et al., 2005; Meyerdierks et al., 2005) and ANME-2/greigite-bearing DSS consortia (Reitner et al., 2005a, b). These microorganisms form differentiated microbial mat complexes that are typically associated with the Black Sea carbonates, which may lead to the formation to chimney-like microbial reefs of up to several meters (Michaelis et al., 2002; Reitner et al., 2005b). Their microbiology and physiology has been investigated by several studies (Pimenov et al., 1997; Michaelis et al., 2002), but little is known about the exact metabolic pathways. Recent phylogenetic and biochemical studies have suggested that the ANME-archaea have supposedly reversed the methanogenic pathway. Current models suggest that methane is converted by methanotrophic archaea to carbon dioxide and reduced by-products (possibly including molecular hydrogen), which are subsequently consumed by sulfate-reducing bacteria (Hoehler et al., 1994). Most of the genes associated with the methanogenesis were identified in ANME-1- and ANME-2-groups (Hallam et al., 2003; 2004). Additionally, Krüger et al. (2003) found a conspicuous Ni-protein in the microbial reef structures, which is similar to the final enzyme of archaeal methanogenesis (methyl coenzyme M reductase - MCR) and most likely involved in the AOM. MCR catalyzes the key step of methanogenesis in archaea, namely the reduction of methyl coenzyme M (CH_3S-CoM) with coenzyme B (HS-CoB) to methane and heterodisulfide CoM-S-S-CoB

(Hindenberger et al., 2006) in the presence of coenzyme F_{430}, a Ni-porphinoid. The reaction takes place under strictly anaerobic conditions. MCR has a molecular weight of 300 kDa and it consists of three different subunits in a $\alpha_2\beta_2\gamma_2$ stochiometry and contains 2 moles per mol of the Ni-porphinoid factor F_{430} (Ellefson et al., 1982; for structure of the cofactor F_{430} see Pfaltz et al., 1982). To date, most of the evidences for the reversed methanogenesis hypothesis based on the analysis of the biofilm genomes (Hallam et al., 2003, 2004) or purification of the enzyme obtained from a protein extract of the whole microbial mat (Krüger et al., 2003). In this study, we used the immunogold labeling technique to show that the expressed enzyme could be detected in both ANME-1 and ANME-2 archaea.

2.2 Materials and Methods

2.2.1 Microbial mat description

Microbial mat samples were collected in 2001 during a cruise with the Russian R/V "Professor Logachev" from the methane seep area located on the NW´ Shelf region (Crimean Shelf) in the Black Sea. The mat samples derived from different methane-related carbonate build-ups were collected by using the manned submersible "Jago" from aboard the R/V "Professor Logachev". The samples described here were taken from the GHOSTDABS-field (44°46´51´´N, 31°59´57´´E) between water depths of 130 – 230 m. A detailed description of the structure and formation of the carbonate towers and the microbial mat community composition has been published by Reitner et al. (2005a, b). The cross section of such a carbonate tower shows distinct layers of different microbial mat types. From each layer samples were taken and prepared for further analyses. Samples intended for immunogold labeling were chemically fixed with 4 % v/v formaldehyde-solution in 50 % phosphate buffered saline (50 mM potassium phosphate supplemented with 70 mM NaCl and 1.4 mM KCl). The samples were kept refrigerated until further processing.

2.2.2 Growth conditions of control strains

Methanothermobacter marburgensis (DSM 2133) and *Methanothermococcus thermolithotrophicus* (DSM 2095) were grown autotrophically as described (Schönheit et al., 1980; Huber et al., 1982). *Methanolobus tindarius* (DSM 2278) was grown heterotrophically (König and Stetter, 1982). Growth was followed by determination of the photometric optical density (light wavelength: 560 nm). Chemical fixation was performed under anaerobic conditions by addition of 0.3 % (v/v) glutardialdehyde and 0.5 % (v/v) formaldehyde to the growing culture. The cells were then harvested and washed three times in 50 mM potassium phosphate buffer containing 0.9% (w/v) sodium (PBS). The cell pellets were embedded in molten agar. Further processing was performed as described below in “Embedding and transmission electron microscopy”. Crude extracts of unfixed cells for Western blotting experiments were prepared as described (Hoppert and Mayer, 1990).

2.2.3 Western blot analysis

Approximately 100 mg (wet mass) of the microbial mat were suspended in 1 ml 50 mM MOPS/KOH buffer (pH 7.0) and then ultrasonicated for 1 min (UP 200S ultrasound processor, amplitude 70 %). The supernatant was centrifuged for 10 min at 10.000xg and then ultracentrifuged for 30 min at approximately 35000xg. The resulting supernatant was used for further analysis. Gel electrophoresis was performed according to Laemmli (1970) with sodium dodecyl sulfate/mercaptoethanol on 14% (w/v) discontinuous polyacrylamide gels. The separated proteins were visualized by silver staining. Polyclonal antibodies were obtained after purification of MCR as essentially described by Hoppert and Mayer (1990) by immunization of rabbits following established protocols. From several polyclonal antisera tested so far, antiserum raised against the MCR from *Methanococcus vannielii* showed highest intensity after immunoblotting analysis (see below) and was selected for immunolocalization procedures. Immunoblotting was performed according to Hawkes (1986) with some modifications. The gels were blotted to PVDF-membrane (Immobilon^{TM}-P^{SQ} membrane, 0.2 µm microporous polyvinylidene fluoride) at 8°C, 90mA overnight in 25 mM Tris (pH 8,3), 192 mM glycine, 20% MeOH. Blots were blocked for 1 h at room temperature with 2% (w/v) BSA in PBS. Afterwards, the membranes were incubated for 2 h at room temperature in PBS with 1% (w/v) BSA and antibody solution (5µl/ml).

Membranes were washed three times (5 min each wash) with 0.05% (w/v) Tween 20 in PBS. Then, the membranes were incubated for 1 h in PBS supplemented with 1% (w/v) BSA and peroxidase-conjungated goat anti-rabbit-antibodies (at a 1:500 dilution), and washed again with 0,05% (w/v) Tween 20 in PBS (three times, 5 min each), followed by two washing steps in PBS for 10 min. Blots were developed in peroxidase substrate-buffer for 30 min. In a final step the membranes were washed two times for 1 min in PBS.

2.2.4 Embedding and transmission electron microscopy

Prior to dehydration and embedding, samples were chemically fixed in 0.3 % (v/v) glutardialdehyde and 0.5 % (v/v) formaldehyde. Dehydration was performed according to established protocols (Roth et al., 1981; Hoppert and Holzenburg, 1998). In brief, pieces of apprx. 1mm^3 volume from distinguishable zones of the microbial mat were dehydrated in a graded ethanol series (15%, v/v, 30% for 15 min; 50%, 75%, 95% for 30 min; 100 % for 1 h) under concomitant temperature reduction to -40 °C. The samples were infiltrated with Lowicryl K4M resin (50%, v/v, in ethanol for 1 h; 66%, v/v, for 2 h; 100 % for 10 h), then polymerized for 24 h at -40 °C and for three days at room temperature. Ultrathin sections of the embedded sample were labeled with the specific antibodies to detect the antigen-containing cells in the microbial mats. For that purpose, the ultrathin sections were placed on drops of 50 mM potassium phosphate buffer containing 0.9% (w/v) sodium (PBS) with the primary antibody directed against coenzyme M reductase and incubated for 90 min. Dilutions down to 1/100 of the original antiserum were suitable for the experiments. In order to remove unbound antibodies, the grids were incubated three times for 5 min on drops of PBS containing 0.05% (w/v) Tween, and then for 5 min on a drop of PBS without Tween. Then, the grids were incubated for 45 min on a 1:80 dilution of a goat-anti-rabbit IgG-gold (Sigma-Aldrich Corp., Taufkirchen) as a secondary marker. Rinsing on drops of PBS containing 0.05% (w/v) Tween was repeated three times followed by a washing step on drops of double-distilled water for some seconds. This step removes any residual buffer salts that may cause precipitates during staining. Grids were then dried and post-stained with 3% (w/v) phosphotungstic acid (pH 7.0) for 3 min.

Electron microscopy was performed in a Philips EM 301 transmission electron microscope at 80 kV and calibrated magnifications. For enhancement of gold particles depicted on electron micrographs, digital images were processed as described (Hoppert and Holzenburg, 1998).

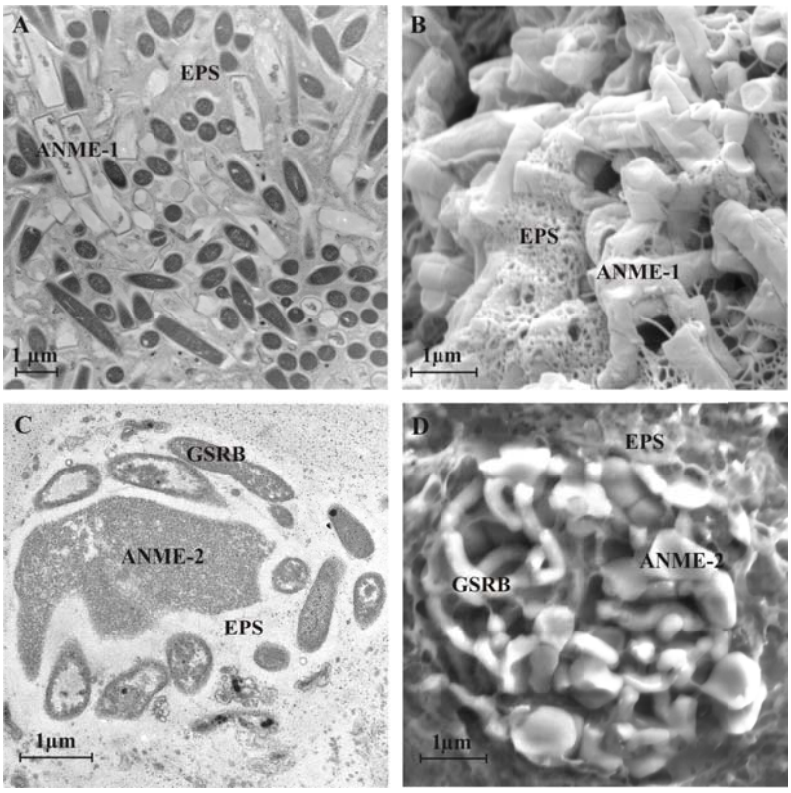

Figure 8: Microbial inventory of the orange-coloured and black layer **a)** TEM imaging of ANME-1 cells in the orange-coloured layer (EPS – extracellular polymeric substances). **b**) Three-dimensional arrangement of ANME-1 cells and their empty sheaths. FE-SEM image of a native (unsputtered) Peldri II-dried sample. **c)** TEM imaging showing aggregates of ANME-2 archaeal group and associated greigite-bearing DSS consortia (GSRB) in the black layer type. **d**) Three-dimensional arrangement of ANME-2 and associated greigite-bearing vibrioform DSS (GSRB). FE-SEM image of a native (unsputtered) Peldri II-dried sample. The network represents remains of extracellular polymeric substances (EPS).

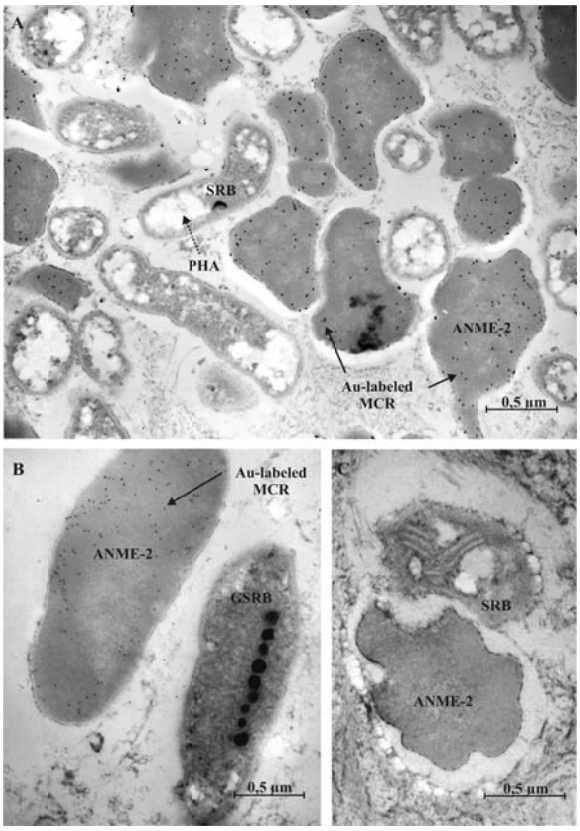

Figure 9: TEM imaging showing localization of MCR in ANME-2 archaea of the black layer. **a, b)** Immunocytochemical localization of MCR in ANME 2 archaea (black dots – Au-labeled MCR). Sulfate-reducing bacteria are not labeled (bright inclusions within these cells are PHA, dark particles in B represent greigite inclusions). c**)** Negative control with preimmune serum. The lighter rim around the cells shows that the cells are shrunken due to fixation.

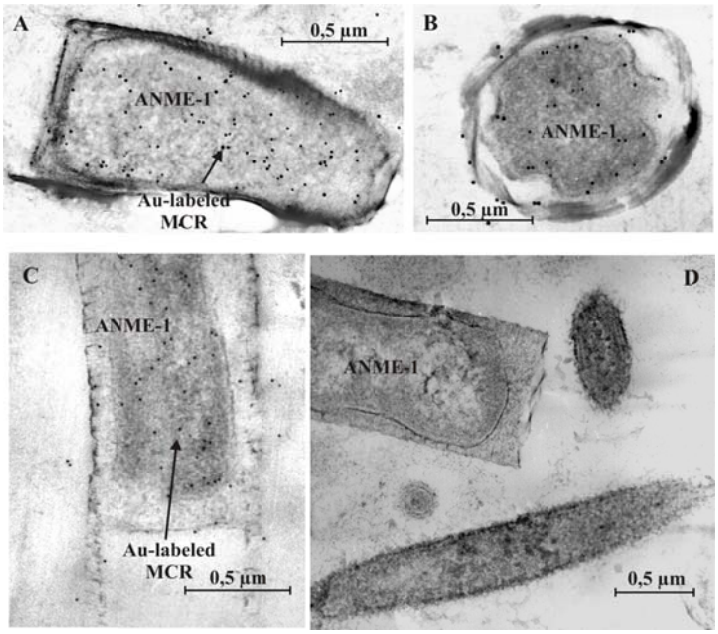

Figure 10: Immunocytochemical localization of MCR in ANME-1 cells in the orange-coloured layer (post-embedding immunogold labeling). **A-C)** Localization of MCR in ANME-1 (black dots – Au-labeled MCR); A and C represent longitudinal sections, B a cross section of the ANME-1 cell type. **D)** Negative control with preimmune serum (PBS).

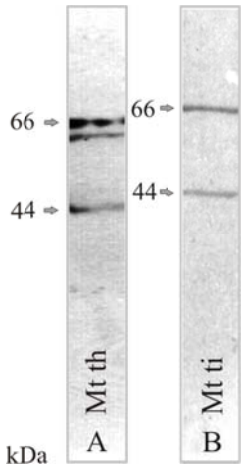

Figure 11: Detection of MCR in the control methanogenic archaea using polyclonal antibody. MCR consists of three different subunits in $\alpha_2\beta_2\gamma_2$ stochiometry (α - 66 kDa, β - 44 kDa and γ 30 - kDa). Grey arrows – subunits of the MCR. **Slot a)** Western blot of the MCR in the *Methanococcus thermolithotrophicus* (Mt th).**Slot b)** Western blot of the MCR in the *Methanolobus tindarius* (Mt ti).

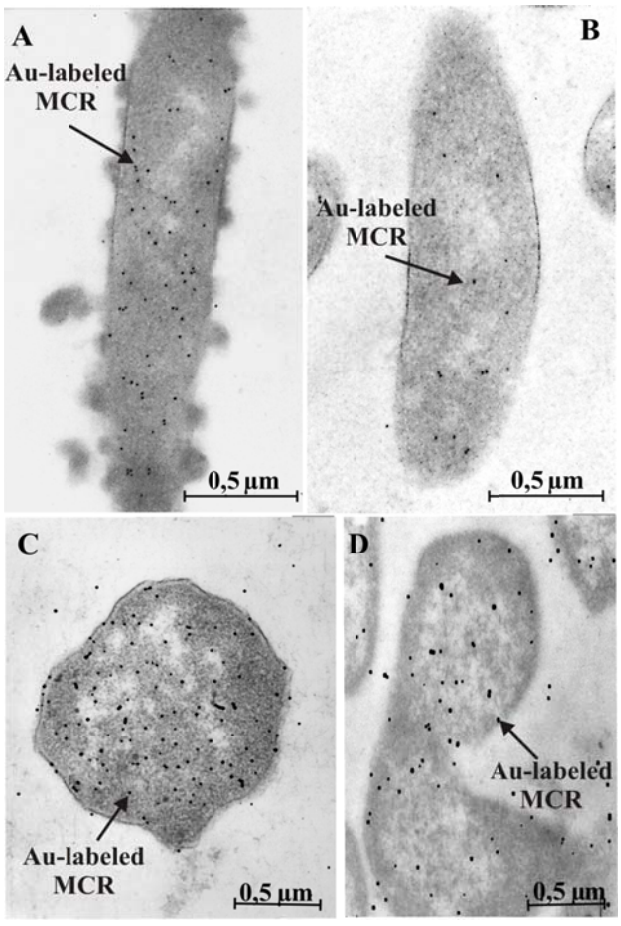

Figure 12: Control experiments with methanogenic archaea **a, b)** Immunocytochemical localization of MCR in *Methanothermobacter marburgensis*-cells during the logarithmic (A) and and stationary phase of growth (B). During the stationary phase, the expression level of MCR decreases. **c, d**) Localization of MCR in *Methanothermococcus thermolithotrophicus* and *Methanolobus tindarius*.

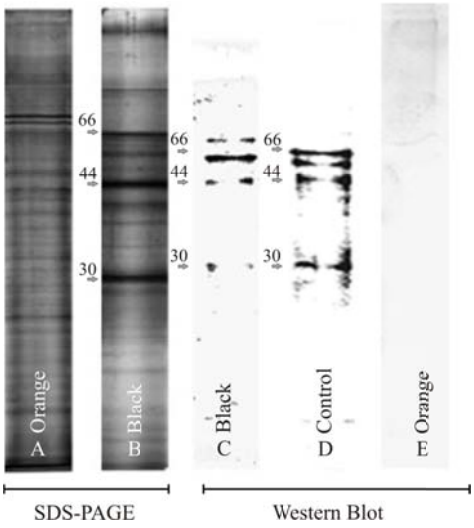

Figure 13: Extracts of the cold seep microbial mats separated by SDS-PAGE and detection of MCR in the microbial mats using a polyclonal antibody. MCR consists of three different subunits in $\alpha_2\beta_2\gamma_2$ stochiometry (α - 66 kDa, β - 44 kDa and γ 30 - kDa). Grey arrows – subunits of the MCR. **Slot a)** Protein extract of the orange-coloured layer. **Slot b)** Protein extract of the black layer. **Slot c)** Western blot of the MCR in the black layer (ANME-2). **Slot d)** Western blot of the MCR in the *Methanothermobacter marburgensis* (Mt mb). **Slot e)** Western blot of the MCR in orange-coloured layer (ANME-1).

2.2.5 Scanning electron microscopy

Samples for the field emission scanning electron microscopy (FE-SEM) were fixed with 4% buffered glutardialdehyde and post fixed with 2% OsO_4, too. After that the samples were dried with Peldri II (Pelco, USA) and HMDS (hexamethyldisilazane; Polysciences, USA) to avoid drying artefacts. Peldri II and HMDS are chemical alternatives to critical point drying. Details of the procedure are described in Brown (1993).

2.3 Results and Discussion

A close-up view on a cold seep microbial reef revealed a distinct internal structure. The microbial reefs were formed by several distinct layers of microbial mats. Figure 8 shows the microbial inventory of the black and orange-coloured layer. As described by Reitner et al. (2005b), the outer surface of the microbial reef is covered by a black layer, which is in direct contact to the permanently anoxic water of the Black Sea. These few millimetre thick layer consists of consortia of archaea and bacteria, which were identified by *in situ* analysis with specific oligonucleotide

probes (Reitner et al., 2005 b). This technique revealed that the bacteria are sulfate reducers (SRB) of the *Desulfosarcina/Desulfococcus* group and that the archaea are affiliated to the *Methanosarcinales* (ANME-2 group) (Pimenov et al., 1997; Michaelis et al., 2002; Tourova et al., 2002; Blumenberg et al., 2004). One remarkable feature of the SRB is the presence of droplets resembling storage inclusions of polyhydroxyalkanoates (PHA, Fig. 9a). Reitner et al. (2005b) have shown the presence of the PHA corroborated by staining with the lipophilic dye Nile blue A. These results agree with the very recent discovery of PHA in members of the DSS-group, including *Desulfosarcina variabilis* and *Desulfococcus multivorans* (Hai et al., 2004). Some SRB contain greigite inclusions (Fig. 8 c, d; greigite bearing DSS consortia, Reitner et al., 2005 a,b). The black layer is followed by an orange-coloured layer and an innermost greenish layer. The orange-coloured layer is composed of the ANME-1 archaea. DAPI-staining and transmission electron microscopy (TEM) shows cylindrically shaped microorganisms with external sheaths (Reitner et al., 2005b). Recent studies have suggested that these microorganisms of the microbial reef layers have supposedly reversed the methanogenic pathway (Hallam et al., 2003; 2004; Krüger et al., 2003). The different analyses have shown that the specific key enzymes of the methanogenesis are encoded in the genomes of the ANME-organisms. One of these enzymes is the methyl coenzyme M reductase, which contains 2 moles per mol of a Ni-compound that appears to be the Ni-cofactor F_{430} of the MCR (Krüger et al., 2003). From genome as well as from biochemical analysis, it was, up to now, not possible, to assign the enzyme to distinct organisms in the microbial mats. In our investigations, the immunogold labeling technique was used to answer the question if the expressed methyl coenzyme M reductase protein could be detected in distinct organisms of the mat and control organisms from pure cultures (Figures 8, 9, 10, 11, 12).

Table 1: Gold particles per square micrometer of cytoplasm compared to extracellular polymeric substances. Localization of gold-labeled antibodies against the methyl coenzyme M reductasein the black and orange-coloured layer of the microbial mats, and in *Methanococcus maritaludis* and *Methanolobus tendaris.*

Layer of the microbial mat	gold particles per square micrometer	
	Cytoplasm	EPS (Extracellular polymeric substances)
ANME-1(orange-coloured layer)	85	6
ANME-2 (black layer)	73	6
Methanothermococcus thermolithotrophicus	98	10
Methanolobus tindarius	37	8
Methanothermobacter marburgensis (log. phase)	90	8
Methanothermobacter marburgensis (stat. phase)	18	4

For that purpose, protein extracts as well as thin sectioned resin-embedded cells were treated with antibodies directed against methyl coenzyme M reductasefrom *Methanococcus vannielii*. In Western blot experiments, only protein extracts obtained from the black layer show the typical band pattern of MCR (Fig. 12, slot C). In blots from the orange-coloured layer, no MCR could be detected (Fig. 12, slot E). This may be explained by the observations obtained by electron microscopy and immunolocalization: the orange-coloured layer consists of more than 90% empty ANME-1 sheaths, i.e., all cytoplasmic contents were already decomposed before collection of the samples. The remaining ANME-1 cells are filled with labeled cytoplasmic contents i.e. they contain MCR and were still alive at the time of sampling (Fig. 10). The total quantity of MCR in these remaining cells are however, obviously too low to result in an MCR-specific signal in the Western blot analysis. In order to quantify the differences in the methyl coenzyme M reductase distribution, the different layers of the microbial mat, gold particles on surface areas of randomly selected organisms were counted and compared with results from control experiments with other methanogenic archaea (Table.1). Like in the microbial mats, the antibodies specifically react against the typical MCR band pattern, when crude extracts of the organisms were used for the blotting experiments (Fig. 11). The density of immunogold markers (i.e. the expression level) in ANME-1- as well as ANME-2-cells is in the order of magnitude of metabolically active cells from logarithmic growth phases of pure cultures. Figures 5 A and B show the different expression levels of MCR during and after the

logarithmic growth phase of *Methanothermobacter marburgensis* cells. During the stationary phase, the expression level of MCR decreases by a factor of five (Fig. 12 b). Though in various methanogenic archaea (Fig. 12 a, c, d), the density of gold markers differ, with respect to the expression level of MCR and the specific antigen-antibody interaction, the density of immunogold markers in all cold seep-samples indicate that the ANME-archaea are as metabolically active as cells taken from the logarithmic growth phase of cultures.

In conclusion, the results show that the methyl coenzyme M reductase is not only encoded in the genomes of the Cold seep-archaea but also expressed at high levels. Immunolocalization reveals a high density of these metabolically active cells in the black layer of the microbial mat. This supports other recent findings: Nauhaus et al. (2007) mentioned that the measured high content of the putative reversed methyl coenzyme M reductase ein the microbial mats may be necessary to compensate the kinetic limits of the first step of anaerobic oxidation of methane. Recent analyses suggested that methane activation is the reversal of the exergonic final step in the methanogenic pathway and, therefore, endergonic: CH_4 + CoM-S-S-CoB → CoM-CH_3 + HS-CoB, ΔG0 around +30kJ mol-1 (Shima and Thauer 2005; Nauhaus et al. 2007). Thus, this reaction appears to be a limiting "bottleneck" for AOM. Though, in contrast to cultivated methanogenic bacteria, the growth yield of ANME-organisms is low, the organisms have high cytoplasmic contents of (reverse) MCR similar to the MCR contents of methanogenic archaea under optimal growth conditions. The high contents of the (reverse) MCR in the ANME-archaea may compensate for this kinetic disadvantage to a certain extent (Nauhaus et al. 2007).

Acknowledgements

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Chapter 3

Nickel signatures as a geochemical indicator for the anaerobic oxidation of methane in recent and ancient microbial mats

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Manuscript

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Abstract

The anaerobic oxidation of methane (AOM) plays an important role in marine basins worldwide. Methane-derived carbonate-build-ups e.g. found on the NW´ Black Sea shelf are part of a unique microbial ecosystem. Two archaeal guilds are mainly responsible for the AOM: ANME-1 (anaerobic-methane-oxidizing communities)/DSS consortia and ANME-2/greigite-bearing DSS-consortia. These microorganisms constitute a significant sink of methane on earth, but despite their relevance for the global carbon cycle little is known about the metabolic pathway of AOM. The nickel-containing MCR is one of the prominent key enzymes of the AOM. Immunogold labeling experiments have shown that the activity of this enzyme depends on the composition of the microbial mat.

Here we present LA-ICP-MS data and stable carbon isotope values determined from recent cold seeps from the Black Sea and the fossil seep of Montepetra (Northern Apennines, Italy), which show specific enrichment patterns for nickel. Due to enzymatic activities, the formation of nickel-containing iron sulfides and the incorporation into the crystal lattice of carbonates, nickel can be accumulated in microbial derived carbonates and the associated mats. Therefore, we will show that nickel concentrations together with ^{13}C-depletion could be a good geochemical indicator for the anaerobic methane oxidation in recent calcified and fossil seeps at a scale down to several mm.

3.1 Introduction

In a wide range of modern and ancient geological environments the precipitation of carbonate minerals are induced by microbes and their metabolic activities (Riding et al., 2000; Krumbein et al., 2003, Aloisi et al., 2006). One of these processes is the anaerobic oxidation of methane (AOM) that is often found to be coupled to sulfate reduction (Reeburgh, 1980). Recent studies have shown that methane might also be oxidized anaerobically with electron acceptors other than SO_4^{2-} (Raghoebarsing et al., 2006; Ettwig et al., 2008; Ettwig et al., 2010; Beal et al., 2009). Nevertheless, sulfate-dependent AOM carried out by consortia of methanotrophic archaea and sulfate-reducing bacteria is very likely to be the most important process in marine environments (e.g. Knittel and Boetius, 2009) In the cold seeps from the Black Sea two archaeal guilds mediate the anaerobic

oxidation of methane: (1) ANME-1 (anaerobic methane-oxidizing archaea)/DSS (Desulfosarcina/Desulfococcus group) consortia and Crenarchaeota (Boetius et al., 2000; Hinrichs et al., 1999; Michaelis et al., 2002; Orphan et al., 2001a, 2001b, 2002; Knittel et al., 2005; Meyerdierks et al., 2005) and (2) ANME-2/greigite-bearing DSS consortia (Reitner et al., 2005a, b). Here these microorganisms were arranged in three different microbial mats: (1) a black microbial mat at the outer surface of the structures; (2) an orange-colored mat which underlies the black mat and (3) a green microbial mat at the inner surface of the carbonate sphere (Reitner et al., 2005b), which is not part of this study. The activity of the different microorganisms leads to alkalinization and causes the precipitation of methane-derived, $\delta^{13}C$-depleted carbonates according to the overall reaction: $CH_4 + SO_4^{2-} + Ca^{2+} \rightarrow CaCO3 + H_2S + H_2O$ (Reitner et al., 2005b; Aloisi et al., 2000; Peckmann et al., 2001, Aloisi et al., 2004, Peckmann und Thiel, 2004). Therefore, and due to the permanent anoxic water body, the AOM sites of the Black Sea are characterized by chimney-like structures that can reach several meters in height (Reitner et al., 2005 a,b; Peckmann et al., 2001; Lein et al., 2002; Michaelis et al., 2002). The carbonates consist of two different phases, namely aragonite and high-Mg-calcite (Reitner et al., 2005b). Despite the fact that the exact metabolic pathway of the anaerobic oxidation of methane is unknown, recent phylogenetic and biochemical studies have suggested that the ANME-archaea have supposedly reversed the methanogenic pathway. Most of the genes associated with the methanogenic pathway, like genes of the Ni-containing enzymes, were encoded in the genomes of the ANME-1 and ANME-2- groups, (Hallam et al., 2003; 2004; Krüger et al., 2003). One of these enzymes, the methyl coenzyme M reductase (MCR), catalyzes the final step of the methanogenesis and represents up to 10 % of all cellular proteins (Diekert et al., 1981). Immunogold labeling experiments with a specific antibody against the MCR have shown that the MCR is not only encoded in the genome of the ANME- groups but is also expressed at high levels, particularly in the black microbial mats (Heller et al., 2008). In contrast to the conspicuous carbonate build ups that are extended to the permanent anoxic water body of the Black Sea, the fossil seep of Montepetra (Italy) is characterized by a different type of a methane-derived carbonate structures. The large Tortonian lenticular carbonate body of the Montepetra seep (Ricci Lucchi and D'Onofrio,

1967; Ricci Lucchi and Veggiani, 1967) consists of polygenic breccias, mottled limestones, laminated micrites and calcarenites (Conti et al., 2010). Furthermore, some parts of the structure contain conduits and millimetric veins (Conti et al., 2010) that were refilled by carbonates. There already exists evidence that AOM was performed within the Montepetra seep. Taviani et al. (1994) compared the fossil seep fauna of Montepetra with those from recent methane seeps where AOM is one of the important processes. Furthermore, Peckmann (1999) could extract specific biomarkers from archaea and bacteria typical for methane seeps.

Here we present LA-ICP-MS analyses of both, the relatively recent (as will be shown with U/Th ages) cold seeps from the black sea and the fossil seeps from Montepetra, demonstrating that Ni concentrations vary in the different carbonate phases as well as in the microbial mats. Furthermore, we will show that the correlation of this data together with stable carbon isotopic data could be a geochemical indicator for the anaerobic oxidation of methane in recent and fossil microbialithes.

3.2 Materials and Methods

The Black Sea carbonates and microbial mat samples were taken in 2001 during a cruise with the Russian R/V “Professor Logachev” on the Crimean Shelf in the Black Sea. The samples analyzed here were collected using the manned submersible “Jago” from the GHOSTDABS-field (44°46´51´´N, 31°59´57´´E) in a water depth of about 230 m; for details see Reitner et al. (2005b). Fossil seep samples were collected in 2009 in Montepetra, Emilia Romagna, Italy (43° 55' 50.94"N, 12° 11' 38.52"E). The fossilized carbonate structures were cut into pieces of 4.5 x 2 cm with a thickness of 5 mm at maximum. The microbial mats were separated and embedded in epoxy resin and afterwards cut in thin slices. On these samples line measurements with the Laser-Ablation-ICP-MS were performed. All measurements were done with an ELAN DRC II ICP-MS (PerkinElmer SCIEX). This instrument is combined with a COMPex 110 ArF Excimer-Laser (Lambda Physik) and an optical bench (Geolas Microlas Lasersystem). Furthermore, a microscopic system with a movable X-Y-Z-table (Zeiss, Physiks Instrumente) is attached. For determination of stable carbon isotope ratios, carbonate samples were taken with a microdrill (Department of

Geochemistry, Dr. A. Kronz, Germany) in direct vicinity of the laser line measured with LA-ICP-MS analysis. Stable carbon and oxygen isotope measurements of these samples were carried out at the Center of Geosciences at the Department of Isotope Geology, University of Goettingen, (Prof. A. Pack, Germany) with a Thermo KIEL IV carbonate device linked to a FINNIGAN Delta mass spectrometer. The δ13C values are referred to the PDB scale (precision of values ± 0.02 ‰). All samples were analyzed with a combined gas chromatograph-mass spectrometer (Varian CP-3800 GC coupled to a Varian 1200 quadrupole MS, Agilent Inc.). U/Th-dating was performed on samples from carbonate concretions taken from the GHOSTDABS field. For this, carbonate samples were collected in each phase of the different completely calcified spheres from the outside to the inside of the Black Sea crust with a microdrill. U-series dating of carbonates were performed using the method of Fietzke et al. (2005) and the decay constants after Cheng et al. (2000) at the Low-Temperature-Isotope-Geochemistry-Lab (LTIGC, IFM-GEOMAR-Kiel).

3.3 Results and discussion

3.3.1 U/Th-ages

Various authors could show that the uranium (U)-series is suitable for dating cold seep carbonates (Lalou et al., 1992; Teichert et al., 2003; Watanabe et al., 2008, Bayon et al., 2009, Feng et al., 2010). In our study, 24 phases of pure aragonite (<97% aragonite) and high-Mg-calcite of methane-derived carbonate spheres from the Black Sea were analyzed. Results for U and Th and the determined ages are given in Table 1. Uranium concentrations (^{238}U) varied in the range from 1.3 to 1.9 µg g-1 for the reddish Mg-calcite phase, for the dark Mg-calcite phase the ^{238}U-value is 4.7 µg g-1. In the pure aragonite phase, the 238U-concentration is in the range from 2.1 to 4.7 µg g-1. These values coincide with those reported in the literature for cold seep carbonates from the Black Sea and worldwide (Aharon et al., 1997; Teichert et al., 2003; Watanabe et al., 2008; Bayon et al., 2009; Feng et al., 2010). Thorium (^{232}Th) concentrations are in the range from 1.1 to 147 ng g-1, whereas the highest concentrations were measured in the dark Mg-calcite phase. Compared to the results reported by Feng et al. (2010), the ^{232}Th-concentrations here are in the same range. Due to the low ^{232}Th concentrations in the Black Sea

samples, the uncertainties of ^{230}Th ages should be small. Aragonite, for example, contains only little or no detrital material (Feng et al., 2010), and therefore, the U/Th ages comprise only small uncertainties. The incorporation of detrital sediments into crusts is one of the major difficulties in dating cold seep carbonates (Bayon et al., 2009). Detrital sediments are the major source of initial ^{230}Th, which is often accompanied by larger amounts of ^{232}Th. A further initial source of ^{230}Th is seawater (Lin et al., 1996; Henderson et al., 2001), which also has to be considered when determining U/Th ages. The δ^{234}U is indicative for the source of the incorporated Uranium, e.g. modern seawater has a δ^{234}U value of 145.8 ± 1.7 (Cheng et al., 2000). δ^{234}U values determined here, are in the range between 175 and 181 and are therefore higher than those of modern seawater. This observation is consistent with that reported in the literature (Feng et al., 2010). The incorporated Uranium is therefore likely derived from pore waters, which have a higher Uranium concentration than modern seawater (Feng et al. 2010; Cochran et al., 1986; Teichert et al., 2003). The calculated U/Th ages of the different carbonate phases of the Black Sea cold seep structures ranged from 0.8 to 1.7 ka (Table 2). More precisely, the formation of Mg-calcite phases (1.7 – 0.8 ka) starts earlier than the formation of the pure aragonite (Fig. 14). Due to the fact, that the formation of High-Mg-calcite starts in the outer, black microbial mat and that the aragonite is mainly formed in the inner, orange-colored microbial mat, the U/Th ages suggest that the outer microbial mats were calcified first. Furthermore, the spheres were calcified from the outside to the inside. Youngest U/Th ages are closest to the fluid pathway or in the inner part of these carbonate spheres because these parts were formed by the microorganisms of the orange-colored microbial mat and later by the aragonitic phases.

Figure 14: Calculated U/Th ages of carbonate nodules from a cold seep of the Black Sea. a) Results from an older nodule with youngest ages of 863 years near the conduit and b) a relative young carbonate nodule with ages of 84 to 122 years.

3.3.2 Stable carbon isotopes

Generally, $\delta^{13}C$-values of carbonates lower than -30‰ are typical for methane seep carbonates, due to the isotopic fractionation of methane during AOM (e.g. Peckmann and Thiel, 2004) The aragonitic and high Mg-calcitic phases obtained from the carbonate structures of the Black Sea revealed $\delta^{13}C$-values in a range from -23‰ up to -43‰. The carbonate phases of the Montepetra fossil seep showed $\delta^{13}C$-values ranged from -33‰ up to -45‰. The highest ^{13}C depletion could be found in the refilled vein of the Montepetra sample (Fig. 2). $\delta^{13}C$-values determined here are similar to those reported in the literature (e.g. Terzi, 1992; Peckmann and Thiel, 2004; Conti et al. 2010) and indicate a thermogenic origin of methane ($\delta^{13}C$ of -50‰ to -20‰ vs. VPDB; Whiticar, 1999), or methane that was formed during secondary biodegradation processes of natural oil and gas ($\delta^{13}C$ of -35 ‰ to -25‰; c.f. Roberts and Aharon, 1994). Nevertheless, the stable carbon isotope ratios evidenced that the anaerobic oxidation of methane takes place in both sample locations.

Table 2: U/Th elemental and isotopic composition and resulting U/Th ages for carbonate spheres from the Black Sea.

Sample	Site	Mineralogy of carbonate phase	^{238}U [µg/g]	^{230}Th [pg/g]	$^{230}Th/^{232}Th$ activity ratio	$^{230}Th/^{234}U$ activity ratio	$\delta\ ^{234}U_{(0)}$ [%]	$\delta\ ^{234}U_{(T)}$ [%]	Age (years BP)	$\delta^{13}C_{PDB}$ [‰]
Loga-01-3	channel center	Mg-calcite (reddish)	1.92 ± 0.01	0.297 ± 0.012	39.23 ± 1.69	0.0079 ± 0.0003	177.6 ± 1.8	178.0 ± 1.8	863 ± 36	-28.64
Loga-01-3	channel center	Mg-calcite (reddish)	1.92 ± 0.01	0.294 ± 0.009	36.60 ± 1.30	0.0078 ± 0.0003	177.6 ± 1.8	178.0 ± 1.8	852 ± 28	-28.64
Loga-01-3	channel center	Mg-calcite (reddish)	1.92 ± 0.01	0.294 ± 0.005	39.03 ± 0.68	0.0078 ± 0.0001	177.6 ± 1.8	178.0 ± 1.8	855 ± 16	-28.64
Loga-01-3	channel center	Mg-calcite (reddish)	1.92 ± 0.01	0.300 ± 0.004	37.92 ± 0.64	0.0080 ± 0.0001	177.6 ± 1.8	178.0 ± 1.8	872 ± 15	-28.64
Loga-01-4	rose rim	Mg-calcite (reddish)	1.32 ± 0.01	0.278 ± 0.003	47.43 ± 0.69	0.0107 ± 0.0002	181.3 ± 1.6	182.0 ± 1.6	1179 ± 18	-25.79
Loga-01-4	rose rim	Mg-calcite (reddish)	1.32 ± 0.01	0.277 ± 0.004	45.61 ± 0.71	0.0107 ± 0.0002	181.3 ± 1.6	182.0 ± 1.6	1176 ± 19	-25.79
Loga-01-7	dark micrite center	Mg-calcite (dark)	4.73 ± 0.03	2.000 ± 0.021	2.56 ± 0.04	0.0155 ± 0.0024	178.2 ± 2.6	179.1 ± 2.7	1706 ± 271	-32.44
Loga-85-5-1	big center	>98% aragonite +Mg-calcite	3.65 ± 0.03	0.084 ± 0.001	10.74 ± 0.18	0.0011 ± 0.0000	175.7 ± 2.4	175.8 ± 2.4	122 ± 3	-35.85
Loga-85-5-2	small arm in center	>98% aragonite +Mg-calcite	2.10 ± 0.02	0.050 ± 0.006	7.06 ± 0.85	0.0011 ± 0.0002	179.6 ± 2.5	179.6 ± 2.5	119 ± 17	-35.02
Loga-85-5-3	outer rim	>98% aragonite +Mg-calcite	4.00 ± 0.03	0.070 ± 0.005	5.08 ± 0.36	0.0008 ± 0.0001	180.3 ± 2.1	180.3 ± 2.1	84 ± 8	-35.78
Loga-85-5-4	opposite rim	>98% aragonite +Mg-calcite	3.04 ± 0.03	0.116 ± 0.010	1.57 ± 0.13	0.0010 ± 0.0005	176.6 ± 2.5	176.7 ± 2.5	113 ± 58	-36.02
Loga-85-4-1	fragile rim	90% aragonite +Mg-calcite	2.96 ± 0.02	0.068 ± 0.005	3.80 ± 0.29	0.0010 ± 0.0001	181.2 ± 1.6	181.2 ± 1.6	105 ± 13	-36.18
Loga-85-4-2	center	>98% aragonite +Mg-calcite	3.82 ± 0.03	0.097 ± 0.002	2.47 ± 0.07	0.0009 ± 0.0002	179.8 ± 2.0	179.8 ± 2.0	100 ± 17	-43.01
Loga-85-4 Blase 1-9	at top exit	90% aragonite +Mg-calcite	3.10 ± 0.02	0.063 ± 0.003	6.93 ± 0.38	0.0009 ± 0.0001	177.3 ± 2.1	177.3 ± 2.1	103 ± 7	-36.30
Loga-85-4 Blase 2-1	inner massive on base cut	80% aragonite +Mg-calcite	3.44 ± 0.03	0.102 ± 0.005	1.92 ± 0.10	0.0009 ± 0.0003	179.0 ± 1.6	179.1 ± 1.6	102 ± 30	-36.15
Loga-85-4 Blase 2-4	middle of vertical cut	90% aragonite +Mg-calcite	3.53 ± 0.03	0.098 ± 0.007	15.64 ± 1.19	0.0014 ± 0.0001	178.2 ± 2.0	178.3 ± 2.0	150 ± 12	-40.86
Loga-85-4 Blase 2-5	top of vertical cut	90% aragonite +Mg-calcite	4.04 ± 0.05	0.105 ± 0.010	2.51 ± 0.24	0.0009 ± 0.0002	179.2 ± 2.1	179.2 ± 2.1	103 ± 23	-39.07

3.3.3 Laser ablation ICP-MS

Laser ablation analyzing allowed us to determine the elemental content of the different phases of the methane derived carbonates, as well as the different microbial mats from the Black Sea and the fossil seep structure of Montepetra (Italy). Elemental concentrations were reported in Table 3.

3.3.3.1 Black Sea

The carbonate phases precipitated in the cold seeps of the Black Sea are characterized by a close interfingering of high-Mg-calcite and aragonite. Precipitation of any of these phases depends on the occurrence of the accordant microbial species. The high-Mg-calcite was formed mainly in the black microbial mat, whereas the aragonite was formed primarily in the orange-colored mat (Reitner et al., 2005b). LA-ICP-MS analyses of the methane-derived Black Sea carbonates confirm the close proximity of calcitic and aragonitic phases as described by Reitner et al. (2005b). The close inter-fingering can be illustrated by the alternating concentrations of Magnesium (Mg) and Strontium (Sr), whereby high Mg concentration is representative for high-Mg-calcite and high Sr concentration for aragonite. LA-ICP-MS values are reported in Table 3. High-Mg-calcite of the Black Sea cold seep carbonates shows Mg-concentrations higher than 15000 ppm, whereas the aragonite reveals values ranging up to 2400 ppm. Sr values range from 1450-3050 ppm in the high-Mg-calcite and from 7800-9000 ppm in the aragonite. The concentration of Sr as well as Mg shows only small variations in the different microbial mats.

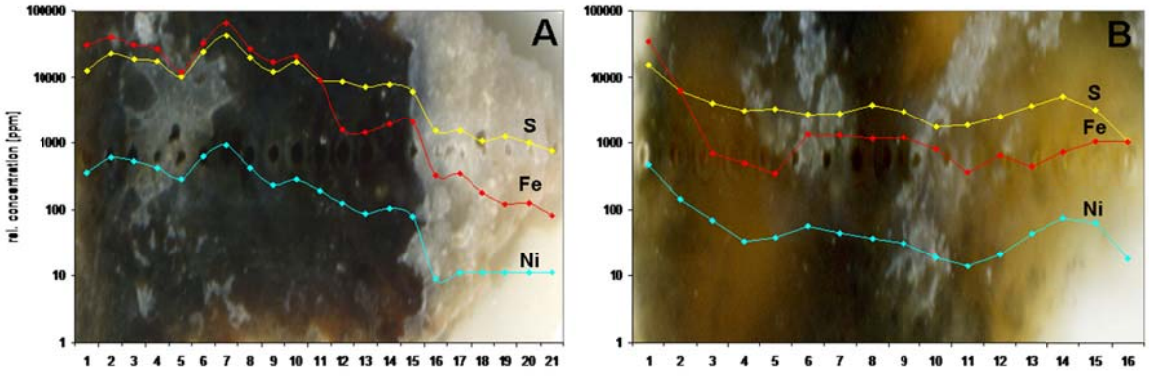

Figure 15: LA-ICP-MS lines for the different carbonate phases of samples from a Black Sea cold seep. a) S, Fe and Ni concentrations in a black microbial mat following by a aragonitic carbonate phase (white part). In the latter, Ni concentration decreased. B) Orange-colored microbial mat. Ni concentrations are lower than in the black microbial mat.

Our main interest was to detect untypical high amounts of Nickel (Ni) in both carbonate phases as well as in the corresponding microbial mats. It has to be noted that there are two main trends concerning the distribution of Ni in the carbonates. First, the Ni concentration in the "mature" methane derived carbonates varies between 1 ppm in the aragonitic phase and 367 ppm in the high-Mg-calcitic phase, whereas the Ni content in the different microbial mats varies from a minimum of 13 ppm Ni in the orange mat type and 1062 ppm Ni in the black mat type. When carbonate precipitation starts in the active microbial mats, Ni concentrations vary from 10 ppm in the aragonites (orange-colored microbial mat) up to 500 ppm in the calcites of the black mat type (Fig.15). Therefore, the highest Ni concentrations are found in the black mat type and in the calcitic carbonates, whereas the lowest concentrations are found in the orange microbial mats and aragonitic phases. The second main feature visible in the line measurements are extremely strong fluctuations of Ni concentrations, visible as single peaks. Those amplitudes often coincide with high concentrations of iron (Fe) and sulfur (S).

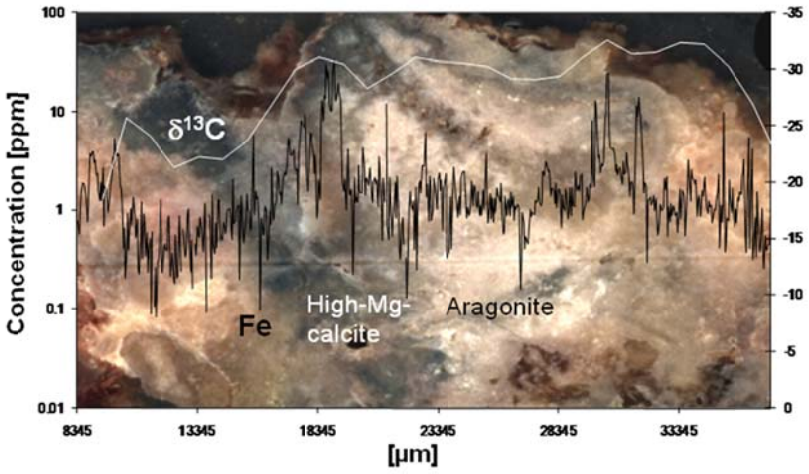

Figure 16: Fluctuations of Ni concentrations, which are visible as single peaks in LA-ICP-MS line measurements.

The reason for the different Ni concentrations in the orange and in the black mat could be linked to the different microorganisms in there. Ni is an important trace element, which can be incorporated into several enzymes. One of these is the MCR, which catalyzes the final methane forming step of methanogenesis in archaea. As described above, the activity of the MCR is much higher inside the black mat. 80 % of the orange mat consists of empty cell membranes (Reitner et

al., 2005b), and therefore the activity of the MCR is much lower here. Hence, the variable Ni concentrations in the microbial mats can be explained by the different activity of enzymes in the mats. Furthermore, the microbial consortia in the mats develop different kinds of exopolymeric substances (EPS), which are made of mainly polysaccharides, proteins, lipids, and nucleic acids (Flemming and Wingender, 2002). The concentration of those components influences the precipitation of either aragonite or calcite. For example, Wada et al. (1993) could show that a higher amount of polysaccharides favors the precipitation of calcite. Therefore, the Ni concentration in the aragonite, which is formed in the orange mat, is lower than the Ni concentration in the calcite, which is formed in the black mat. Additionally, the SRB contain intracellular aggregates of iron sulfides in single crystals or strings. After the cell lysis the iron sulfides are released and accumulate to clusters of framboids. Those framboids are mainly enriched in the high Mg-calcites (Reitner et al., 2005b). Also, Ni is often associated with these iron sulfides. The scattered accumulations of the iron sulfides are illustrated by the amplitudes in figure 16. According to that, these sulfides demonstrate another important accumulation process of Ni in the carbonates and microbial mats (Fig.16). This is in concordance to the depletion of ^{13}C, which shows a higher depletion in regions of high Nickel concentration.

Table 3: Major and trace element concentration for microbial mats and associated methane-derived carbonates (Black Sea) and fossil seep carbonates (Montepetra, Italy).

Sample		Mg [ppm]	S [ppm]	Mn [ppm]	Fe [ppm]	Ni [ppm]	Sr [ppm]
Black Sea carbonates							
001	High-Mg-Calcite	41161	38123	2431	6635	160	2746
	Aragonite	2400	1000	290	150	22	14000
005	High-Mg-Calcite	27187	967	2782	1513	47	1453
	Aragonite	1700	380	150	530	8	9000
002	High-Mg-Calcite	15404	722	2185	368	11	4500
	Aragonite	1600	650	247	680	15	8300
004	High-Mg-Calcite	19379	15995	1157	23570	367	6945
	Aragonite	800	275	123	115	3	8050
006	High-Mg-Calcite	18750	1550	2950	1450	30	3050
	Aragonite	1115	530	165	950	15	7800
003	High-Mg-Calcite	20759	905	2399	255	22	2380
	Aragonite	1450	325	145	115	11	8350
007	High-Mg-Calcite	20216	967	3397	696	32	2337
	Aragonite	474	136	90	94	1	7825
Black Sea carbonate phases inside the microbial mats							
33Y	Calcite	15551	2554	2468	824	33	1215
	Calcite	17743	4725	2282	6322	111	1928
	Calcite	77502	10757	7517	3901	125	1301
33Z	Aragonite	8278	1898	1735	464	21	13162
	Calcite	22648	20698	4548	29915	498	1264
	Calcite	27670	11570	5779	13405	280	1729
	Calcite	37824	7834	6752	10265	238	2229
744-1	Aragonite	4167	1852	1022	466	159	10600
	Aragonite	6137	1438	1012	886	72	15018
	Calcite	37097	2125	7585	933	91	7900
744-2	Aragonite	813	657	122	793	32	9575
	Aragonite	635	421	166	405	10	129655
Black Sea microbial mats							
33Y	orange	3032	3704	64	1593	59	61
	orange	2336	5470	187	5045	124	69
	transition	16857	23941	607	16367	343	106
33Z	black	2821	9876	244	6874	159	85
	black	3858	14486	166	6549	275	95
	black	3202	12813	213	6166	234	102
744-1	orange	2485	2337	28	2986	350	156
	black rim	3844	5910	393	24803	1062	273
	orange	2810	1970	445	1980	186	187
744-2	orange + black	619	536	104	1220	43	3136
	orange and black	585	194	94	302	13	295
Montepetra fossil seep							
MP308	conduit 1	1700-5800	10-900	110-320	10-470	1-2.5	190-750
	conduit 2	3200-5200	15-850	160-310	50-1100	1-5.5	180-350
	dark grey part	3800-8200	15-6000	160-360	100-3500	1-14	230-410
	conduit 4	2480-7700	15-980	120-250	70-2500	1-5.5	250-1270

3.3.3.2 Montepetra

The cold seep of Montepetra consists of a complex carbonate body, which is pervaded by veins and conduits. Those are pathways for rising fluids, where microbial mats can develop. Therefore only the veins and conduits are part of this study. LA-ICP-MS analyses of the carbonates of the Black Sea and the refilled conduits and veins of the Montepetra fossil seeps show a similar distribution for Ni, Fe and S. The nickel concentrations inside the conduits vary between 1 and 15 ppm, whereby the highest concentration can be observed in the dark layer (Fig. 17).

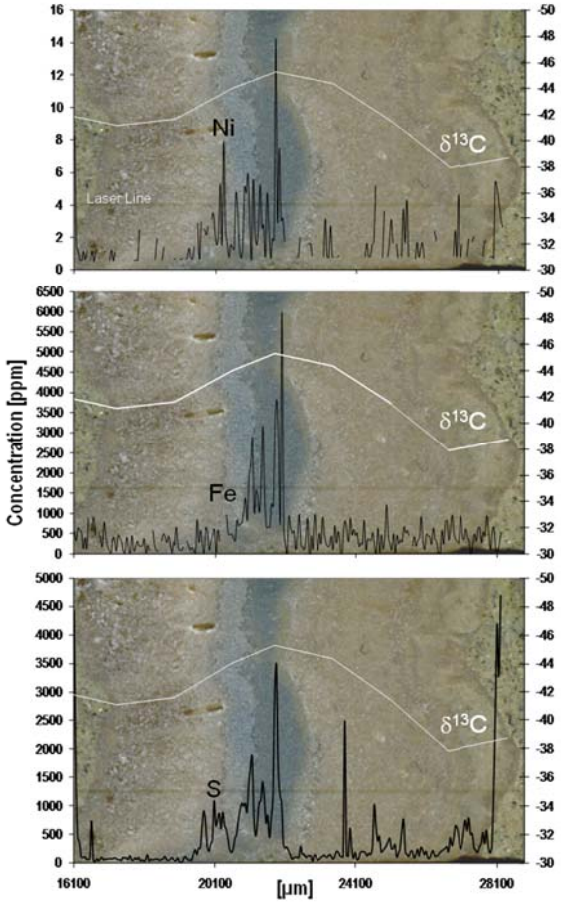

Figure 17: Element concentration of Ni, Fe and S in a conduit of the fossil seep in Montepetra (Italy).

The same applies also for Fe and S, with concentration of 10-3500 ppm Fe and 10-6000 ppm S. Regions with high Ni concentrations in the conduits and veins also show a higher $\delta^{13}C$-depletion. Peckmann (1999) describes lipid biomarker data of the conduits and veins from the Montepetra seep that are indicative for the

AOM. This includes the isoprenoids phytane, PMI, squalane, and biphytanes, which are indicative for methanogenic archaea and short chain fatty acids, especially iso- and anteiso-C_{15}-fatty acid, that are specific for a bacterial source input, in particular from sulfate reducing bacteria (Peckmann, 1999). Altogether the data suggest that the anaerobic oxidation of methane takes place in the veins and conduits.

3.4 Conclusions

In general, high Ni concentration could occur either due to the methanogenesis or AOM, because both metabolic pathways are based on the same enzymes. During methanogenesis, ^{13}C isotope will be enriched, whereas during AOM, ^{13}C gets depleted. Therefore, Ni concentration always needs to be considered together with the stable carbon isotope data. The data presented here from the Black Sea cold seeps and the fossil Montepetra seep show that Nickel is enriched in AOM associated microbial mats. Reasons for this enrichment could be several processes. The occurrence of specific microorganisms which use Ni-containing enzymes for their metabolic activity are one possible reason for the enrichment of Ni, from 2 ppb, the concentration in sea water (Eitinger and Madrand-Berthelot, 2000) to ppm range in living mats and seep carbonates. Furthermore, the formation of Ni-containing iron sulfides which are generated in the SRB and during cell lysis released into the surrounding EPS is an important enrichment factor. Additionally, during precipitation of carbonates Ni is incorporated into the crystal lattice. Ni together with $\delta^{13}C$-values could serve as a geochemical indicator for the AOM in recent and fossil seeps.

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Chapter 4

The expelled mud volcano fluids (gas, water and sediment particles): first attempt

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Manuskript

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Abstract

Terrestrial mud volcanoes in Italy which are part of this study are situated in the Northern Apennines and Sicily and were formed by the expulsion of water and mud as well as gaseous and liquid hydrocarbons. Most of the mud volcanoes worldwide are associated with an active petroleum reservoir. In there, several secondary microbial and diagenetic processes occur. The expelled gases deriving from the reservoirs are dominated by methane, higher hydrocarbons and carbon dioxide and are of early mature thermogenic origin. The higher n-alkanes extracted from the emitted fluids indicate an immature source rock likely mixed with fresh organic matter, confirming the results made by gas analyzes. Carbon stable isotope analyzes of the carbon dioxide provide evidence for biodegradation and secondary microbial methanogenesis taking place in the fluid reservoir. The expelled waters derived from a depth of 2 km are brackish, and provide evidence for the influence of secondary diagenetic processes directly in the reservoirs or during the rise of the fluids. Therefore, the expelled mud volcano fluids derive from deep-situated reservoirs which provide ideal conditions for microbial activities.

4.1 Introduction

Reports of mud volcanism in Italy can be traced far back in history. First of all, Pliny reported in his *Naturalis Historia* (AD 77) about mud volcanism in Italy (Conte 1982). Three areas, in Northern Italy e.g. the Salse di Nirano, central Italy and Sicily e.g. the Maccalube di Aragona (Martinelli and Judd, 2004) are most prominent with respect to mud volcanism in Italy. The arc-shaped Apennines, one of the youngest mountain chains on earth, is a thrust-and-fold belt that was formed during the Neogene and Quaternary in conjunction with the rotation and motion of the Corsica-Sardinia block and the following opening of the Tyrrhenian Sea. The Apennine chain consists of the northern Apennine arc, a stack of northeast-vergent thrust sheets, and the southern Apennine arc (Muttoni et al., 1998; Vai and Martini, 2001). In Northern Italy, mud volcanism is located in the foreland of the Northern Apennines in a zone of tectonic compression (Martinelli and Judd, 2004). Sicily is located along the Eurasia-Nubia convergent plate boundary (Dewey et al., 1989; Serpelloni et al., 2007; Catalano et al., 2008). This collision complex is characterized by three different areas, (1) the Hyblean Foreland outcropping in

southeastern Sicily; (2) the Caltanissetta Basin, a dynamic foredeep basin which was formed from the Late Miocene to the Quaternary and (3) a complex chain thrust towards the east and southeast, consisting of the Calabrian Arc and the Maghrebian thrust belt (Vallone et al., 2008 and references therein). Mud volcanoes in Sicily, e.g. the Maccalube di Aragona and Comitini occur over the accretionary wedge developed in front of the Sicilian–Maghrebian fold-and-trust belt (Madonia et al., 2011). Mud volcanoes emit a multi-phase mixture of gaseous hydrocarbons, fluids, sediments and sometimes higher liquid hydrocarbons (e.g. Milkov, 2000). Usually, the fluids are originated from deep subsurface sediments and are often related to active petroleum systems (Brown, 1990; Kopf, 2002, Milkov, 2000 and Etiope et al., 2009a). Here, a brief introduction into the composition and the sources of the different phases is given. Organo-geochemical, geochemical and stable isotope analyzes were performed to understand the geochemical and microbiological processes that take place in the mud volcanoes and/or the associated reservoirs.

4.2 Methods

4.2.1 Sampling sites of the mud volcano fluids

The gas, water and expelled sediment samples were collected during four campaigns from 2008 to 2010 at different mud volcano sites in Northern Italy and Sicily (Tab. 4.).

Table 4: Sample locations in Northern Italy and Sicily

Sampling Site	Location	Campaigns
Northern Italy		
Salse di Nirano (NR)	N44° 30' 49.68" E10° 49' 24.6"	March 2008, June 2009, March and October 2010
alse di Ospitaletto	N44° 26' 22.56" E10° 53' 20.76"	June 2009, October 2010
Sicily		
Maccalube die Aragona (MAC)	N37° 22' 35.88" E13° 36' 0.18"	March and October 2010
Comitini	N37° 26' 34.32" E13° 39' 6.96"	March 2010
Paternò	N37° 34' 22.45" E14° 53' 24.58"	October 2010

4.2.2 Geochemistry of the water phase

The chemical-physical parameters (pH, Eh, electric conductivity and water temperature) were measured directly in the field. Total alkalinity was determined by acidimetric titration using a hand-held titrator and 1.6 N H_2SO_4 cartridges (Hach Corporation). The water samples were taken with a vacuum pump, connected to a flexible tube with a metal pipe at the open end (UniSampler, Bürkle, Bad Bellingen, Germany) and were filtered through 0.45 µm cellulose syringe filters until the samples were free of particle. One portion for the cation analysis was acidified with 65% nitric acid (HNO_3). An aliquot of un-acidified fluid was kept for anion analysis of the sample. The cation and anion concentration was determined on an ion chromatograph with chemical suppression and conductivity detection (Metrohm) and with an inductively coupled plasma optical emission spectrometer (ICP-OES; PerkinElmer Optima 3300 DV) according to Thomson and Walsh (1983).

4.2.3 Geochemical analyses

The total carbon (TC), nitrogen (TN) and sulfur (TS) concentrations were measured by a CNS elemental analyzer (Euro Vector Instruments and Software, Milano, Italy). The total organic carbon (TOC), after acidification with H_3PO_4, was also determined on a CNS elemental analyzer (Euro Vector Instruments and Software, Milano, Italy). The total inorganic carbon (TIC) was calculated by the difference between TC and TOC. Mineral phase analyses of the freeze-dried fluids were performed on a *Philips X Pert MPD* (X-Ray-diffraction) equipped with a PW3050 Goniometer (Cu as anode material). Data were collected from 4 to 65°2θ using a step size of 0.02°2θ and a count time of at least 2 seconds per step.

4.2.4 Gas sampling and gas chromatography

Gas bubbles were collected in the central part of the different gryphons and pools using a funnel and special gas vials (Labco Vials, Labco Limited, Buckinghamshire, United Kingdom). Analysis of permanent gases and light hydrocarbons were performed on a Varian-GC, equipped with three detectors: one TCD for nitrogen gas (N_2), carbon dioxide (CO_2), hydrogen gas (H_2) and oxygen/argon (O_2/Ar) (Ar and O_2 cannot be separated), saturated and unsaturated hydrocarbons were detected on two FID´s (Middle FID for methane (CH_4), ethane

(C_2H_6), propane (C_3H_8) and butane (C_4H_{10}) and Rear FID for pentane (C_5H_{12}). Carrier gas was helium (He) and the separation of permanent gases and methane was carried out on a Molsieve-13X (1.5 m 1/8"), a Hayesep Q (0.5 m 1/8") and a Hayesep T (0.5 m, 1/8") column. Higher hydrocarbons were separated on a Silicaplot (30 m, 0.32 mm) and a CP-SIL 5CB capillary column. The detection limit for permanent gases was 100 ppm, for molecular nitrogen 500 ppm and for methane 1 ppm. The error of measurement was gas and concentration dependent. For concentrations below 5% the relative error was between 1% and 10%, for concentrations above 5% between 0.3 and 1%.

4.2.5 Isotope ratio-Mass Spectrometry

Carbon and hydrogen isotopic analysis of hydrocarbons were performed on a Finnigan Delta Plus, isotope mass spectrometer coupled to an Agilent gas chromatograph (GC-IRMS). The components were separated on a Poraplot Q column (inner diameter 0.32 mm, length 25 m). Hydrocarbon components were converted to CO_2 in a combustion oven at 940°C for $\delta^{13}C$ measurements and reduced to H_2 (deuterium measurements) in a reduction furnace at 1470°C. $\delta^{13}C$ values are given versus Vienna-PDB and $\delta^{2}H$ versus standard mean ocean waters (SMOW).

4.3 Results

4.3.1 Mineralogical compositions of the fluids

Quartz was the dominant mineralogical compound in all mud volcano fluids. The main carbonate phase was calcite, whereas the accessory minerals were albite as well as the clay minerals chlorite, illite and kaolinite. The contents of total inorganic carbon (TIC) in mud volcano fluids ranged from 1.9 to 3.3%. The total organic carbon (TOC) was in the range of 0.4% to 0.7% (Table 5). The total nitrogen (TN) content was between 0.05% and 0.10%, and the total sulfur (TS) content 0.05% to 0.55%. The carbonate amounts ranges from 20% to 24% in the Northern Italy mud volcanoes and from 11% to 17% in the mud volcanoes of Sicily.

Table 5: Organic and inorganic components of the dried mud volcano fluids (% w/w = percent per weight).

Sample	TC	TOC	TIC	$CaCO_3$	N_{tot}	S_{tot}	C_{org}/N_{tot}	C_{org}/S_{tot}
	mean	mean	calc.	Calc.	mean	mean		
	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
Sicily								
Maccalube di Aragona 1	2.1	0.7	1.5	12.5	0.09	0.19	6.4	3.1
Maccalube di Aragona 2	2.0	0.6	1.4	11.8	0.10	0.32	6.0	1.9
Maccalube di Aragona 3	1.9	0.6	1.3	11.2	0.10	0.29	5.9	2.0
Comitini 2	2.6	0.7	2.0	16.9	0.08	0.55	7.4	1.0
Northern Italy								
Salse di Nirano 4	2.9	0.5	2.4	20.2	0.06	0.25	7.8	1.9
Salse di Nirano 7	3.0	0.4	2.6	21.3	0.05	0.17	8.3	2.5
Salse di Nirano 9	3.2	0.6	2.6	21.6	0.07	0.24	8.6	2.3
Salse di Puianello	3.3	0.4	2.9	24.3	0.05	0.05	8.4	6.9

4.3.2 Geochemical water composition

The results of the pH, Eh, temperature, conductivity, alkalinity, concentration of major and trace elements (i.e., F^-, Cl^-, Br^-, NO_3^-, SO_4^{2-}, K^+, Na^+, Ca^{2+} and Mg^{2+}) are shown in Table 7. The pH values were between 7.5 and 8.2 in the Northern Apennine mud volcanoes, and between 7.6 and 8.5 in the mud volcanoes in Sicily. Thus, mud volcanoes in both areas are characterized by nearly neutral to slightly alkaline pH-values. The Paternò mud volcano had a pH of 6.2, which is slightly acidic. The salinity of the collected water ranged from 12.2 to 16.4 g/kg in Northern Italy and from 13.2 to 20.0 g/kg in Sicily. The Paternò mud volcano had a salinity of 67 g/kg. All mud volcano waters studied here were brackish. Na and Cl were hereto the most abundant elements (Fig. 18). The concentration of Cl was in the range of 6200 to 8100 mg/L in the mud volcanoes of the Northern Apennines and of 5770 to 8800 mg/L in Sicily. The highest concentrations of Cl (~ 41200 mg/L) were found in the Paternò mud volcano, which had also the highest Na concentration (24490 mg/L). Mud volcanoes in the Northern Apennines were characterized by concentrations of 4900 to 7200 mg/L Na. Sulfate concentrations in the waters were generally low (3 – 280 mg/L) compared to seawater (3100 mg/L). Furthermore, the emitted mud volcano waters were enriched in boron (B) and bromine (Br). The observed boron concentration of up to 160 mg/L in the CH_4-

driven mud volcanoes represents a 30-fold enrichment compared to seawater. Waters expelled from Paternò represent a 50-fold enrichment of B compared to seawater.

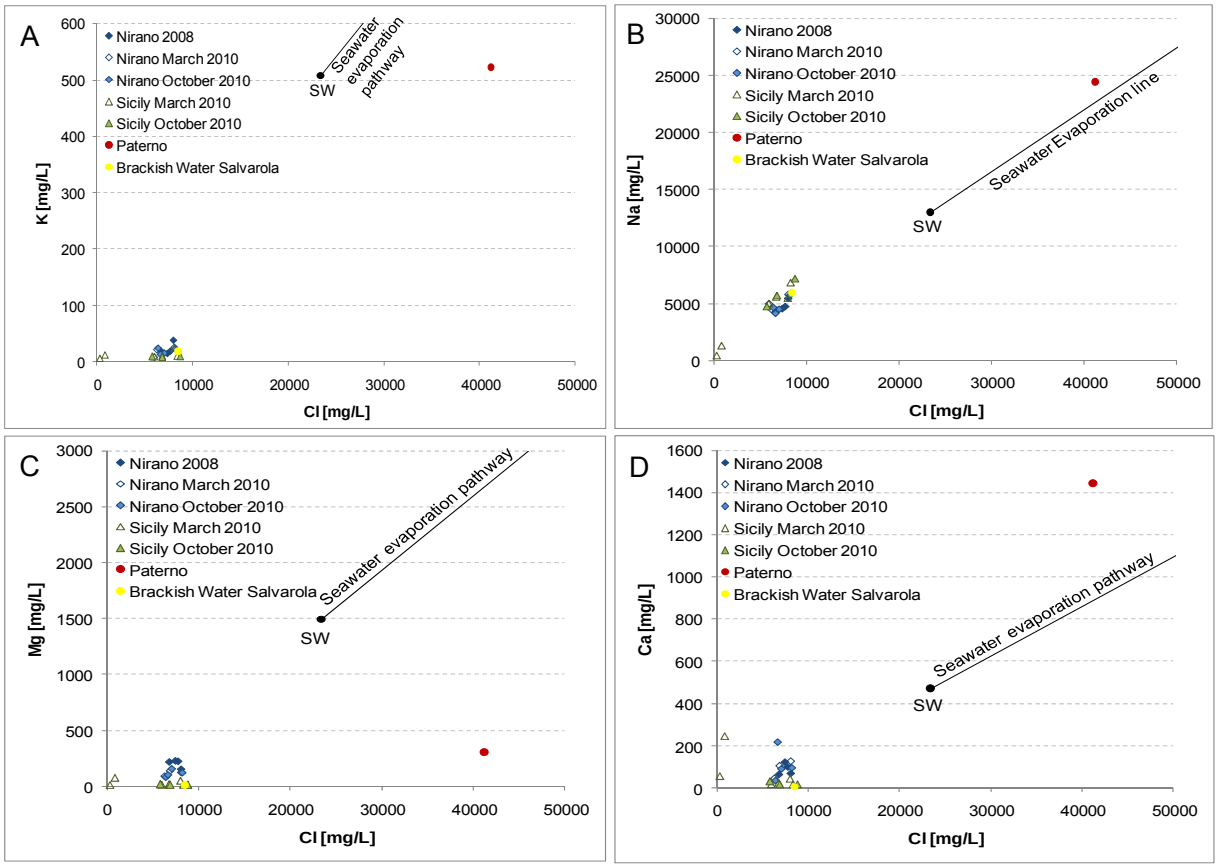

Figure 18: Major element concentrations versus Cl. a) K-conc.; b) Na-conc.; c) Mg-conc.and d) Ca-conc. The seawater evaporation line after Fontes et al. (1993) is also reported.

Geothermometers, based on the temperature-dependent water-mineral exchange, were applied to identify the prevailing temperature and the depth of the fluid reservoirs. Geothermometers based on the K-Na, K-Mg and K-Ca composition were used (after Giggenbach, 1988). The overall calculated reservoir temperatures were 27 to 182°C (Table 6). The lowest temperatures were calculated for the Maccalube di Aragona, the highest values for the Paternò mud volcano. Mapping the Na, K and Mg concentrations of waters onto a Na/1000-K/100-√Mg graph (a combination of K/Mg and K/Na geothermometers according to Giggenbach (1988)), demonstrate that they plot directly on the line representing full equilibrium or in case of the Northern Apennines mud volcanoes in the field of full equilibrium. These waters have most likely reached the thermodynamic equilibrium with the subsurface rocks (Fig. 19).

Table 6: Formation water temperatures calculated according to Giggenbach, (1988).

		K/Na [°C]	K/Mg [°C]
Northern Italy			
Salse di Nirano	August 2008	62	46
Salse di Nirano	August 2008	55	42
Salse di Nirano	August 2008	66	47
Salse di Nirano	August 2008	83	66
Salse di Nirano	March 2010	57	47
Salse di Nirano	March 2011	68	59
Salse di Nirano	October 2010	57	46
Salse di Nirano	October 2011	71	63
Ospitaletto	October 2010	55	48
Sicily			
Maccalube di Aragona	March 2010	33	57
Maccalube di Aragona	March 2010	28	60
Maccalube di Aragona	March 2010	33	56
Maccalube di Aragona	October 2014	30	57
Maccalube di Aragona	October 2015	27	61
Maccalube di Aragona	October 2016	39	57
Comitini	March 2016	65	67
Paternò	October 2017	133	126

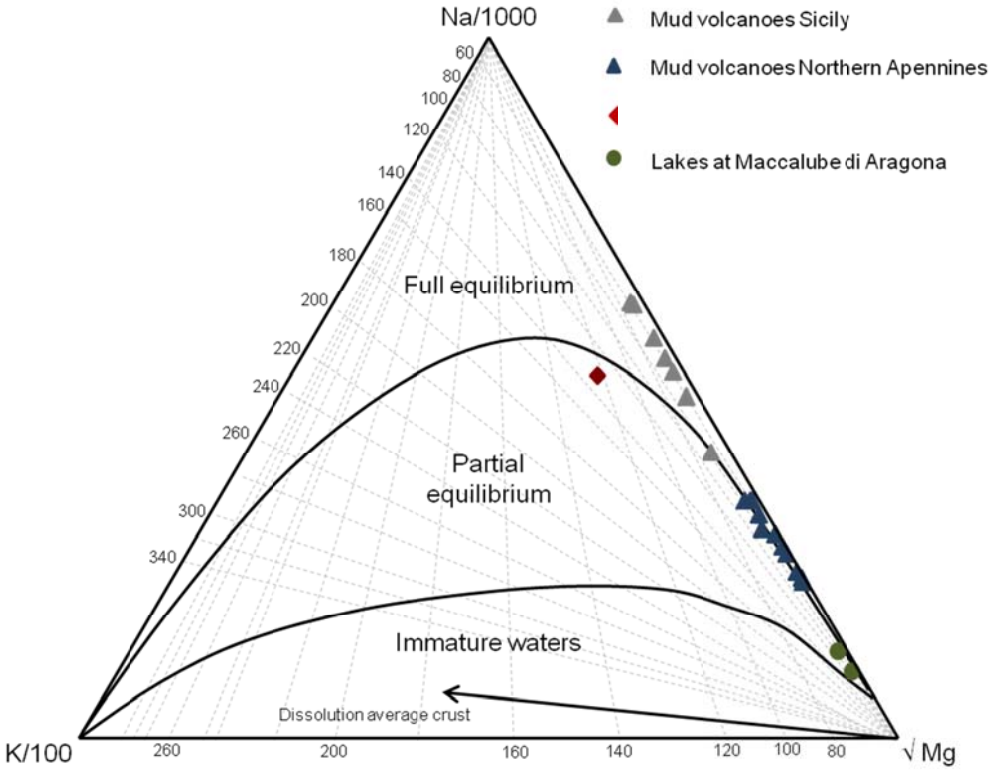

Figure 19: Ternary plot of Na/1000-K/100-√Mg, a combination of K/Mg and K/Na geothermometers (after Giggenbach, 1988), The analyzed mud volcano waters plot on the full equilibrium line or direct in the full equilibrium field, only the Paternò mud volcano waters plot into the partial equilibrium field. The waters are characterized by temperatures below 80 °C, whereas Paternò waters are characterized by a temperature around 130 °C.

Table 7: Physico-chemical parameters and major element concentrations in the waters of mud volcanoes in the Northern Apennines and Sicily.

	Campaign	Salinity	pH	Eh	Ca	Mg	Na	K	Sr	B	TA	Cl IC	Br IC	SO_4 IC	SI Calcite	SI Aragonite	SI Dolomite
		[g/kg]		[mV]	[mg/L]	[mg/L]	[mg/L]	[mg/L]	µg L-1	[mg/L]	[meq/L]	[mg/L]	[mg/L]	[mg/L]	IAP/KT	IAP/KT	IAP/KT
Northern Italy																	
2008																	
Salse di Nirano 2	Jun 08	13.46	-	-	97.5	223.2	4698	18.8	4132	-	11.73	7719	78.7	11.9	5.9	4.3	195.0
Salse di Nirano 4	Jun 08	12.91	7.860	107	120.9	227.0	4512	14.8	5238	-	10.40	7408	77.3	8.6	6.3	4.5	182.0
Salse di Nirano 9	Jun 08	12.19	7.958	-117	61.7	216.9	4279	19.0	4306	-	14.15	6742	71.4	15.1	4.6	3.2	147.9
Salse di Nirano 9	Jun 08	14.91	7.895	11	67.0	151.8	5413	38.5	5140	-	17.20	8043	88.2	193.3	5.9	4.3	199.5
2010																	
Salse di Nirano 4	March 2010	12.38	7.830	-	102.9	135.3	4418	15.2	7740	-	14.08	6858	64.9	4.8	4.7	3.3	45.7
Salse di Nirano 9	March 2010	12.57	8.210	-	44.2	88.2	4484	21.1	5176	-	27.46	6274	60.1	8.0	9.5	6.8	331.1
Salse di Puianello	March 2010	16.29		-	123.9	121.1	5767	24.9	27536	-	36.20	8099	62.6	13.1	4.8	3.4	36.3
2010																	
Salse di Nirano 4	Oct 2010	12.84	7.894	43	88.3	156.1	4518	15.6	5326	118.1	14.55	7076	65.5	3.5	4.5	3.1	52.5
Salse di Nirano 9	Oct 2010	13.01	8.093	37	32.2	79.8	4676	24.3	3990	131.5	27.08	6426	61.2	21.6	4.9	3.4	95.5
Salse di Ospitaletto	Oct 2010	11.92	7.562	183	217.3	100.2	4156	13.4	32083	15.4	11.70	6666	51.1	3.8	4.3	3.0	12.3

	Campaign	Salinity	pH	Eh	Ca	Mg	Na	K	Sr	B	TA	Cl IC	Br IC	SO_4 IC	SI Kalzit	SI Aragonit	SI Dolomit
		[g/kg]		[mV]	[mg/L]	[mg/L]	[mg/L]	[mg/L]	[µg/L]	[mg/L]	[meq/L]	[mg/L]	[mg/L]	[mg/L]	IAP/KT	IAP/KT	IAP/KT
Sicily																	
2010																	
Maccalube di Aragona 1	March 2010	13.87	8.5	-	14.9	14.7	4980	8.1	4735	-	45.74	6001	19.7	149.0	8.3	5.9	128.8
Maccalube di Aragona 2	March 2010	18.76	8.2	-	14.2	14.6	6820	9.3	6634	-	60.70	8368	27.1	13.5	5.8	4.1	72.4
Maccalube di Aragona 3	March 2010	15.31		-	22.1	21.6	5560	9.1	5370	-	47.00	6808	22.3	138.2	6.3	4.6	83.2
Comitini	March 2010	14.82	8.0	-	41.1	51.0	5456	23.6	8200	-	20.86	8055	33.5	8.0	4.9	3.5	63.1
2010																	
Maccalube di Aragona 1	Oct 2010	15.69	8.5	66	15.4	15.0	5661	8.2	4099	122.5	49.33	6845	22.0	134.4	8.9	6.3	151.4
Maccalube di Aragona 2	Oct 2010	19.98	8.4	-60	15.5	15.0	7163	9.6	6059	155.6	66.80	8757	28.2	8.3	9.5	6.8	169.8
Maccalube di Aragona 3	Oct 2010	13.20	8.3	-33	29.4	20.0	4688	9.4	3857	102.4	38.93	5778	18.0	278.2	9.3	6.6	112.2
Paternò	Oct 2010	66.89	6.2	217	1442.7	303.4	24490	522.4	186884	243.9	25.15	41205	101.3	85.9	2.1	1.5	2.5
Seawater*		-	8.2	-	471	1494	13007	508	-	5	2	23397	78	3072	-	-	-

*Seawater concentration of Mediterranean Sea (Madonia et al., 2011)

Table 8: Gas composition and gas ratios of the mud volcanoes (MV) and mud pools located in Northern Italy and Sicily

Sample location	Campaign		N_2 [Vol.-%]	O_2 + Ar [Vol.-%]	CO_2 [Vol.-%]	CH_4 [Vol.-%]	C_2H_6 [ppm]	C_3H_8 [ppm]	i-C_4H_{10} [ppm]	n-C_4H_{10} [ppm]	i-C_5H_{12} [ppm]	n-C_5H_{12} [ppm]	C_1/(C2+C3) [ppm]
Sicily												---	
Maccalube di Aragona	10/2010	pool 1	33.92	9.50	0.51	55.99	790	0	0	0	0	---	708
Maccalube di Aragona	10/2010	pool 2	35.35	9.86	0.41	54.27	1027	59	0	0	0	---	500
Maccalube di Aragona	3/ 2010	pool 3	11.99	3.43	0.73	83.74	1080	47	0	6	---	---	743
Maccalube di Aragona	3/2010	MV	6.12	1.87	1.10	90.18	6186	810	0	257	---	---	129
Comitini	3/2010	MV	5.47	2.12	0.68	91.62	1024	15	0	3	---	---	882
Paternò	3/2010	MV	19.13	5.60	71.03	4.20	404	10	0	0	0	---	102
Northern Italy													
Salse di Nirano	3/ 2010	MV 4	7.13	2.42	0.66	89.73	537	5	0	1	---	---	1657
Salse di Nirano	3/ 2010	MV 4	<0,00002	---	0.32	80	---	---	---	---	---	---	
Salse di Nirano	3/ 2010	MV 9	2.29	0.83	0.73	96.09	532	11	0	1	---		1769
Salse di Nirano	6/2009	MV 7	9.52	3.26	0.24	86.87	729	259	44	11	1	3	879
Salse di Nirano	8/ 2008	MV 7	---	---	0.48	<100	---	---	---	---	---	---	
Salse di Ospitaletto	10/ 2010	MV	42.53	11.87	0.58	45.01	158	2	0	0	0	---	2807

Table 9: Stable carbon and hydrogen isotopic compositions of gases sampled at mud volcanoes in Northern Italy and Sicily (campaigns 2008-2010).

Sample Location	Campaign		C_1/ Sum Cn	$C_1/(C_2+C_3)$	C_2/C_1	i / n C_4	i / n C_5	δ^{13}-CH_4 (VPDB)	δ^{13}-C_2H_6 (VPDB)	δ^{13}-C_3H_8 (VPDB)	$\delta^{13}CO_2$ (VPDB)	δD-CH_4 (SMOW)
Siciliy												
Maccalube di Aragona	10/ 2010	Pool 1	0.9986	708	0.0014	---	---	-47.6	-21.0	---	10.8	---
Maccalube di Aragona	3/ 2010	Pool 1	0.9948	200.1	0.0045	---	---	-46.5	---	---	-15.6	---
Maccalube di Aragona	10/ 2010	Pool 2	0.9980	500	0.0019	---	---	-46.0	-14.4	---	3.9	---
Maccalube di Aragona	3/2010	Pool 2	0.9980	503.5	0.0019	---	---	-46.3	-22.7	---	-4.2	-189.7
Maccalube di Aragona	10/2010	Pool 3	0.9967	300	0.0032	---	---	-50.0	-23.6	---	-9.6	---
Maccalube di Aragona	3/2010	Pool 3	0.9986	743.2	0.0013	---	---	-46.9	-22.8	-12.5	11.5	-179.5
Maccalube di Aragona	3/2010	MV	0.9920	128.9	0.0069	---	---	-46.7	-25.6	-22.6	12	-171.5
Comitini	3/2010	MV	0.9989	881.8	0.0011	---	---	-45.3	-22.1	---	13.1	-176.4
Paternò	10/2010	Pool 2	0.9903	102	0.0096	---	---	-35.7	-23.6	---	0.5	---
Northern Italy												
Salse di Nirano	10/2010	MV 4	0.9994	1814	0.0006	---	---	-44.8	-14.3	---	-4.2	---
Salse di Nirano	3/2010	MV 4	0.9994	1656.6	0.0006	---	---	-44.8	-15.2	---	13.9	-183.2
Salse di Nirano	6/2009	MV 4	0.9990	999.5	0.0008	10.62	0.00	-38.7	-16.0	-14.4	-2	
Salse di Nirano	3/2008	MV 4						-53.9				-167.9
Salse di Nirano	10/2010	MV 9	0.9993	1497	0.0006	---	---	-45.9	-15.2	---	3.7	---
Salse di Nirano	3/2010	MV 9	0.9994	1768.7	0.0006	---	---	-45.8	-15.4	---	15.6	-179.8
Salse di Nirano	6/2009	MV 9	0.9998	6895.3	0.0001	3.55	3.03	-45.2	-15.7	-14.2	8.2	
Salse di Nirano	8/2008	MV 9						-47.3				-187.8
Salse di Nirano	6/2009	MV 7	0.9988	878.7	0.0008	3.87	0.41	-45.3	-18.1	-16.2	1.2	
Salse di Nirano	3/2008	MV 7						-49.5				-175.1
Salse di Puianello	10/2010	MV	1.0000		0.0000	---	---	-15.5	---	---	-14.4	---
Salse di Puianello	3/2010	MV	0.9911	111.4	0.0088	---		-41.4	-21.1	-4.6	25.5	-188.0
Salse di Puianello	6/2009	MV	0.9995	2127.0	0.0005	---	---	-43.00	---	---	---	---
Salse di Ospitaletto	10/2010	MV	0.9996	2807	0.0004	---	---	-46.8	-19.7	---	17.2	---
Salse di Ospitaletto	6/2009	MV	0.9997	4216.0	0.0002	---	0.58	-44.0	---	---	6.1	

4.3.3 Molecular compositions of the emitted mud volcano gases

Gas samples were collected with a funnel at the surface of the different mud volcanoes and pools and were analyzed for gas compositions by gas chromatography. The results are reported in Table 8. Methane was the dominant gas in most of the mud volcanoes. Furthermore, small amounts of higher hydrocarbons (C_{2+}), carbon dioxide and nitrogen gas were apparently also released. It has to be noted that the gas composition in the Salse di Ospitaletto mud volcano seems to be contaminated by air. Carbon dioxide was the main gas component released from the mud volcano of Paternò. Furthermore, nitrogen, small amounts of methane and trace amounts of the higher hydrocarbons (C_{2+}) were also expelled.

4.3.4 Isotopic compositions of the released gases

Table 9 presents the carbon stable isotope ratios of methane emitted at the different mud volcanoes. The Northern Apennines mud volcano gases were characterized by $\delta^{13}C\text{-}CH_4$ values in the range from -50 to -39‰. The $\delta^{13}C\text{-}CH_4$ values of mud volcano gases in Sicily ranged from -50 to -35‰. In both areas, the $\delta D\text{-}CH_4$ values varied between -189 and -167‰. Figure 20a presents a $\delta^{13}C\text{-}CH_4$ versus $\delta D\text{-}CH_4$ diagram according to Whiticar et al. (1999) and Milkov et al. (2010; 2011), which also contains the gas generation fields. According to this, an early mature thermogenic origin is likely for all mud volcano gases. A further distinction between thermogenic and microbial origin of the gas is possible by plotting $\delta^{13}C\text{-}CH_4$ versus the ratio of methane to the heavier methane homologues $[C_1/(C_2+C_3)]$-ratio (Bernard et al., 1976). Figure 20b presents a respective diagram including genetic fields defined according to Whiticar et al. (1999) and Milkov et al. (2010; 2011). Compared to the C-D diagram, all mud volcano samples plot into the genetic field of biodegraded and secondary microbial gas, indicating mostly thermogenic origins, which endured biodegradation.

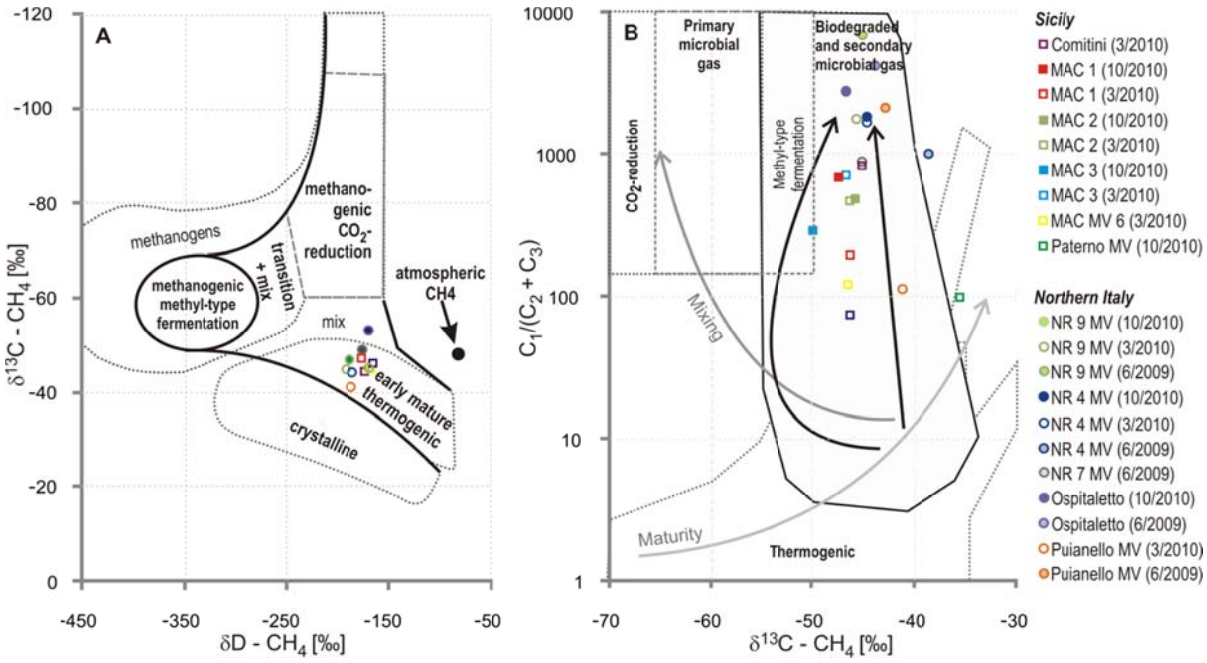

Figure 20: a) Stable carbon and hydrogen isotopic compositions of released methane gas. Genetic fields were generated according to Whiticar et al. (1999) and Milkov (2010; 2011). b) Diagram after Bernard et al. (1976). Genetic fields were generated according to Whiticar et al. (1999) and Milkov (2010; 2011).

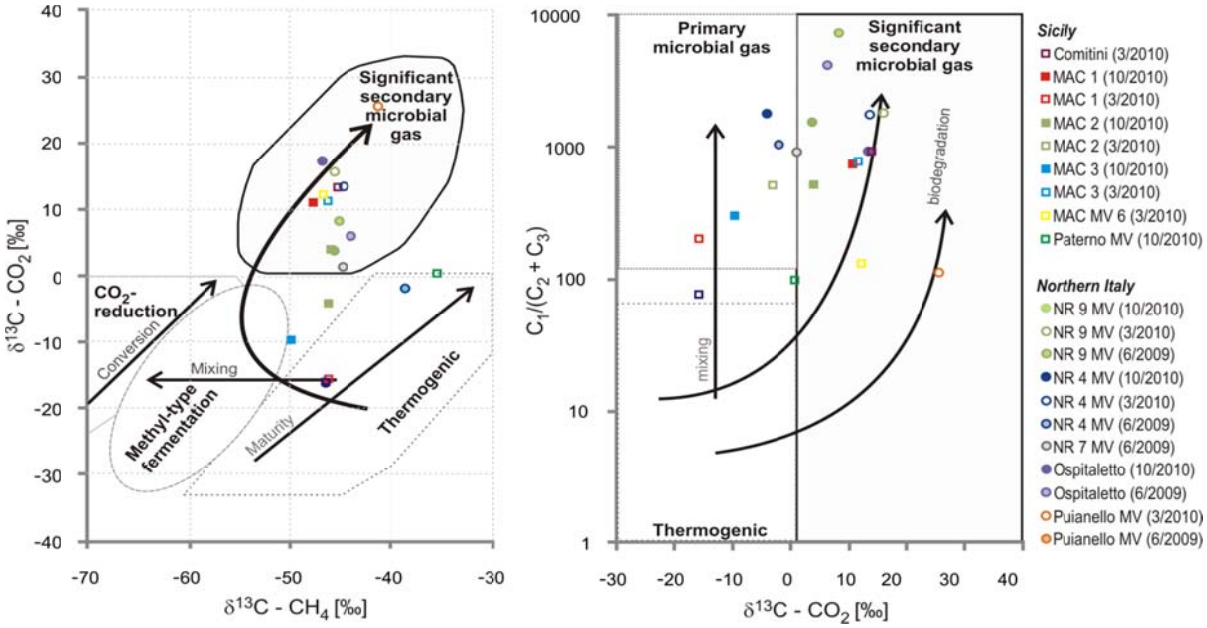

Figure 21: a) Stable carbon isotopic composition of released methane and carbon dioxide gases. Genetic fields were generated according to Whiticar et al. (1999) and Milkov (2010; 2011). b) Diagram after Bernard et al. (1976); "Bernard" ratio versus carbon dioxide. Genetic fields were generated according to Whiticar et al. (1999) and Milkov (2010; 2011).

4.3.5 Isotopic composition of carbon dioxide

Mud volcanoes emit also small fractions of carbon dioxide which is often enriched in ^{13}C (Etiope et al., 2009b). In Northern Italy the $\delta^{13}C\text{-}CO_2$ values were found to range from -14‰ to +26‰ and in Sicily from -16‰ to +13‰. Interestingly, gas from one mud volcano collected at different times was characterized by different $\delta^{13}C\text{-}CO_2$ values, e.g. in a Salse di Nirano mud volcano $\delta^{13}C\text{-}CO_2$ values was +13.5‰ in March 2010 and -4‰ in October 2010 (Tab. 4). A cross plot of $\delta^{13}C\text{-}CH_4$ versus $\delta^{13}C\text{-}CO_2$ is shown in Figure 21a. Obviously, most of the gases plot into the genetic field of the significant secondary microbial gas origin (according to Whiticar et al. 1999; Milkov et al. 2010; 2011). The “Bernard” diagram is shown in Figure 21b. Cross plot of $\delta^{13}C\text{-}CO_2$ versus the “Bernard ratio” $[C_1/(C_2+C_3)]$ revealed that most of the gases are generated due to secondary microbial methanogenic and/or biodegradation processes.

4.3.6 Distribution of higher hydrocarbons (C_{15+})

The distribution of higher *n*-alkanes and the isoprenoid hydrocarbons 2,6,10,14-tetramethylpentadecane (pristane) and 2,6,10,14-tetramethylhexadecane (phytane) is shown for the mud volcanoes in both areas, the Northern Apennines (Salse di Nirano) and Sicily (Maccalube di Aragona and Comitini) by *m/z* 57 mass chromatograms in Figure 22. The higher hydrocarbons in the mud of the Salse di Nirano mud volcano sampled in 2009 (Fig. 22a) were dominated by *n*-alkanes with carbon chains from C_{17} to C_{35} (maximum at C_{29}/C_{31}) and a strong odd-over-even predominance. In the low molecular weight range, a modal distribution was observed (Heller et al. 2011). The Maccalube di Aragona mud pool, Sicily, revealed a bimodal distribution (carbon chain lengths from C_{14} to C_{35}) with maxima at C_{19} and C_{29} (Fig. 22b). The Comitini mud volcano was dominated by *n*-alkanes with carbon chain length from C_{17} to C_{35} (maximum at C_{31}, and a strong odd-over-even predominance and a modal distribution) (Fig.22c). Pristane and phytane were abundant in all samples.

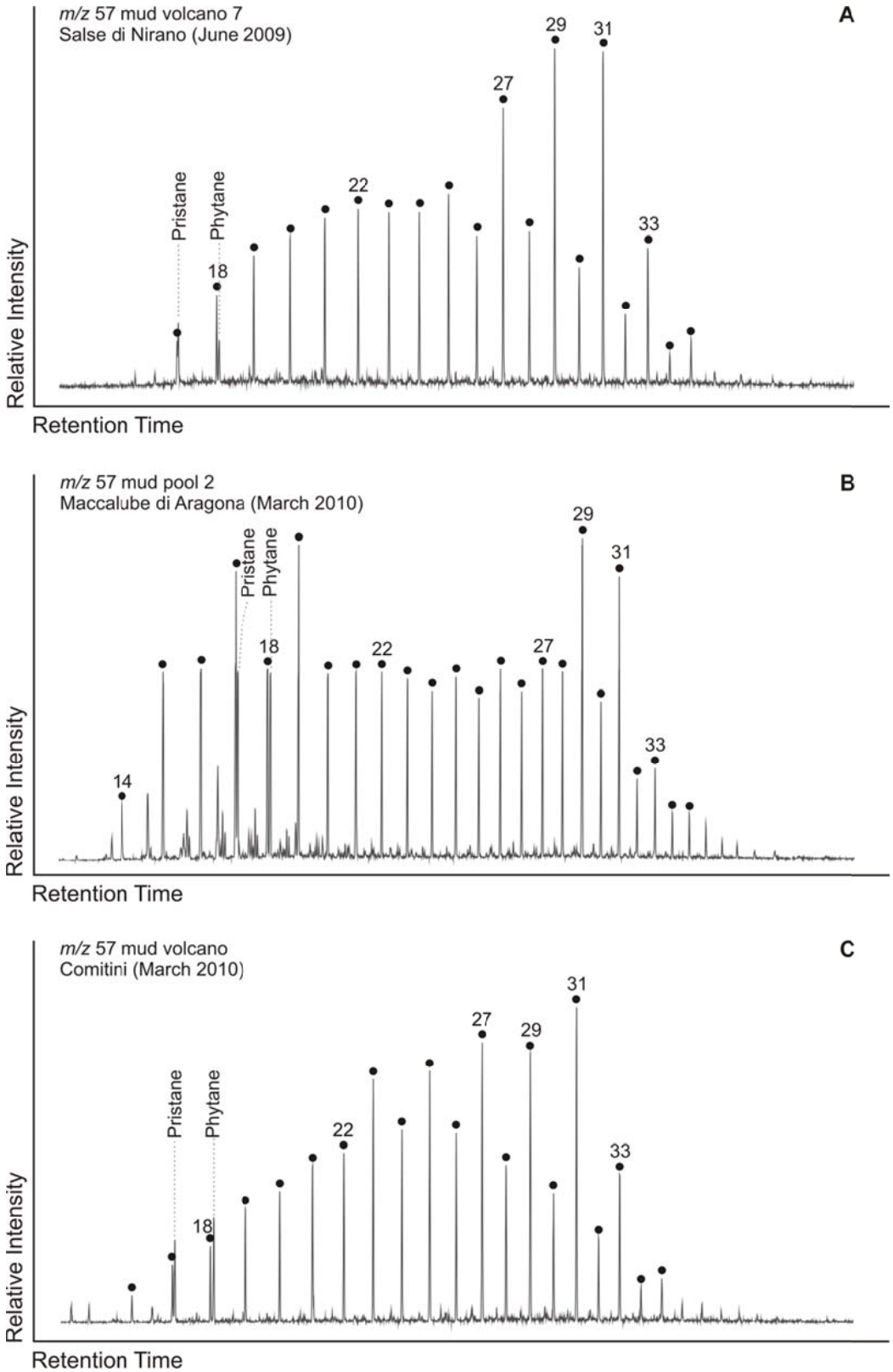

Figure 22: Distribution of *n*-alkanes, pristane and phytane extracted from the mud volcanoes of (A) Salse di Nirano (Northern Italy), (B) Maccalube di Aragona (Sicily) and (C) Comitini (Sicily). The saturated hydrocarbons are highlighted by m/z 57 ion chromatograms.

4.4 Discussion

Mud volcanism in Italy caused by tectonic stress results in emission of fluids composed of gaseous and liquid hydrocarbons, sediment particles and water (Fig. 23). Most of the mud volcanoes are associated with an active petroleum reservoir (Etiope et al. 2009b). Therein, several secondary microbial and diagenetic processes occur (Wenger et al., 2002). Typically, petroleum (oil and gas) is trapped in porous and permeable sediments (sandstones and limestones) filling 80% of the pore space with water making up the rest (Head et al., 2003). In a second part of the reservoir, below the oil-saturated leg, the pore space is filled with nearly 100 % water of variable salinities. All of these waters are essential for biological activities and biodegradation of the oil and gas in the reservoirs (Head et al., 2003). These and the water of the surrounding sediments were squeezed out and expelled at the mud volcanoes, enriched/mixed with liquid and gaseous hydrocarbons and sediment particles. All three phases were analyzed to get insights into the source of the fluids.

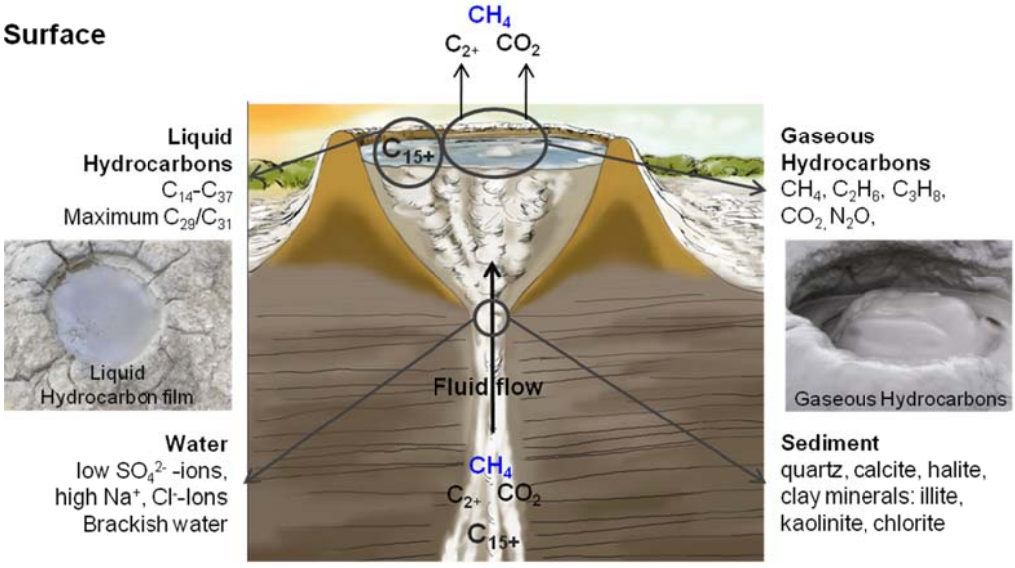

Figure 23: Results of the emitted mud volcano fluid (water, gaseous and liquid hydrocarbons and sediment particle) using the example of the Salse di Nirano mud volcano cone.

4.4.1 Water geochemistry of the emitted mud volcano fluids

The mud volcano waters in the studied areas in Italy are generally brackish and have chemical compositions similar to those of springs and groundwaters in the Emilia-Romagna sector of the Northern Apennines (Duchi et al., 1955; Conti et la., 2000; Boschetti et al., 2010). Those waters can be formed by mixing of evaporated

seawaters and meteoric waters, which is, however, unlikely for mud volcano waters. Mixing of marine connate waters trapped in the pore space of the source rocks with a third diagenetic end-member is more likely as origin for the mud volcano waters. This third end-member is formed by secondary diagenetic processes such as dolomitization-chloritization, zeolitization-albitization and illitization (Boschetti et al., 2010), which modify the major and trace element concentration of the water. Results can be an enrichment of Ca and Sr and a depletion of K, Mg and Na compared to the seawater evaporation path (Fontes et al., 1993; Boschetti et al., 2010). To gain information about sources of the mud volcano waters, the major and trace element contents were analyzed. Compared to seawater the Cl concentration in the mud volcano waters is depleted (Fig. 18), which can be the result of processes such as meteoric water intrusions, clay dehydration and membrane filtration (Gieskes et al., 1989; You et al., 2004). Moreover, all fluids are depleted in K (Fig. 18a), which could be explained by illitization of smectites and/or ion-exchange reactions between waters and minerals (Hower et al. 1976; Martin et al., 1996). Ca and Mg are also depleted compared to seawater (Fig. 18c, d), which can be caused by intense water-sediment exchanges at high temperatures as observed in other mud volcanoes (You et al., 2004) and/or secondary Ca- and Mg-bearing carbonate precipitation (Madonia et al., 2011). Sulfate is also depleted compared to seawater, caused by bacterial sulfate reduction associated with degradation of organic matter and/or anaerobic oxidation of methane (Murray et al., 1978; Capozzi et al., 2002) that takes place in the associated petroleum reservoirs or during the rise of the fluids. Therefore, it is likely that the mud volcano fluids were formerly marine connate waters from the underlying marine sediments, which were squeezed out by tectonic compression and were accumulated in deep-seated reservoirs. Inside these reservoirs or during the migration to the surface the properties of marine connate waters were modified by diagenetic processes. Similar observations were also made for many other mud volcanoes and associated pore-waters (Dia et al., 1999; You et al., 2004; Martin et al., 1996; Brown et al., 2001). Compared to the waters that were expelled from the CH_4-derived mud volcanoes, the waters emitted from the mud volcano of Paternò can be described as brine (salinity of 67‰). In general, major (Na and Cl) and trace element concentrations found in the

waters of the Paternò mud volcano are generally higher than Mediterranean seawater (Table 7). Furthermore, Ca, Mg and Na plot near the seawater evaporation path, which shows that the brines of the Paternò mud volcanoes are evaporated marine connate waters.

The triangular Na-K-Mg graph demonstrates that most of the emitted water has reached their thermodynamic water-mineral equilibrium with reservoir temperatures below 80°C in the CH_4-driven mud volcanoes and a temperature of 130°C for the mud volcano of Paternò. These temperatures suggest that the sources of the emitted waters are located in a depth of 2 to 3 km based on a thermal gradient of 23°C/km for the Northern Apennines (Capozzi and Picotti, 2002) and 20°C/km observed for the southern Sicily foredeep (Mattavelli and Novelli, 1990). Furthermore, temperatures below 80°C provide ideal conditions for microbial activities such as biodegradation of hydrocarbons and secondary methanogenic processes (Wenger et al., 2002).

4.4.2 Gas generation

A helpful tool to distinguish the different formation pathways of gas are stable isotope signatures. Therefore, the molecular composition and isotopic ratios of carbon and hydrogen of the emitted gas (methane, ethane, and propane and carbon dioxide) were analyzed. The $\delta^{13}C\text{-}CH_4$ values in the range from -50 to -35‰ and the $\delta^{13}C\text{-}CH_4$ vs. $\delta D\text{-}CH_4$-diagramm confirm a thermogenic origin of the discharged gas. This is in accordance with data reported in previous studies (e.g. Mattavelli et al., 1987, Etiope et al., 2009). Post-genetic processes, however, such as biodegradation of petroleum and/or secondary methanogenesis (Etiope et al., 2009b) most likely modified the molecular and isotopic composition of the gas (Fig. 20 and 21) and make it difficult to identify the generation process of the gas. Those processes can occur directly in the fluid reservoir or during the migration of the fluids. To better assess these potential reactions, the carbon isotopic values were plotted in a modified “Bernard” diagram ($\delta^{13}C\text{-}CH_4$ vs. $[C_1/(C_2+C_3)]$; Bernard et al., 1976) with the genetic fields according to Milkov et al. (2010, 2011)(see Fig. 20 b). The diagram shows that most of the released gases run through secondary microbial processes e.g. biodegradation and secondary methanogenesis. These processes occur when, (1) gas is biodegraded or is associated with biodegraded

oil accumulations, (2) the associated carbon dioxide has $\delta^{13}C\text{-}CO_2$ values > 2‰, (3) methane gases have $\delta^{13}C$ values in the range of -55‰ to -35‰ and (4) the gas revealed a relatively high dryness ("Bernard" ratio > 50) (Milkov et al., 2011). $\delta^{13}C\text{-}CO_2$ values up to +26‰ confirm that the released gases and/or that the associated petroleum reservoirs was/were considerably influenced by biodegradation and secondary methanogenic processes. The large temporal variability of $\delta^{13}C\text{-}CO_2$ found inside one mud volcano (e.g. Salse di Nirano) and the absence of isotopically enriched carbon dioxide in some mud volcano cones, however, do not rule out that the mud volcano system was affected by biodegradation processes. In fact, it shows that among other controls, stable carbon isotopes of CO_2 are variable and can be affected by various water-rock interactions (Etiope et al. 2009b). But, a positive $\delta^{13}C\text{-}CO_2$ value > 10‰ suggests indeed the presence of biodegradation and secondary methanogenic processes (Pallasser et al., 2000; Etiope et al., 2009b), values that were observed at many studied sites. Based on this, it is most likely that biodegradation and secondary methanogenesis takes place in the mud volcano systems.

4.4.3 Higher hydrocarbons (n-alkanes C_{15+})

Beside the gaseous hydrocarbons, all studied mud volcanoes contain high amounts of liquid hydrocarbons (carbon chains C_{14}-C_{35} with a maximum at C_{29}/C_{31}). All show moderate to strong odd-over-even predominance. Moreover, the Salse di Nirano (Northern Apennines) and Comitini (Sicily) mud volcanoes show a modal distribution in the low molecular weight range. The predominance of *n*-alkanes with carbon chain lengths of C_{27}, C_{39}, C_{31} deriving from immature source rocks with a high input of land plants, and modal distribution patterns in the low molecular weight range suggest a mixture of early thermogenic and less mature hydrocarbons, while the latter most likely were extracted by the rising fluids from organic-rich rocks and sediments (Heller et al., 2011b).

4.5 Conclusion

Mud volcanism in Italy can be found all along the Apennine chain and in Sicily, and the individual mud volcano systems show strong similarities. Most of them are associated with active petroleum systems. As known from the Salse di Nirano the main reservoir of the associated petroleum system is located in a depth of 2 km

and a second shallower one is located in a depth of 200 m. The application of geothermometers has shown that the source of the gas, liquid hydrocarbons and the water is located in a depth of 2 to 3 km confirming the results from other studies. The gas deriving from the reservoirs has an early mature thermogenic origin, which became apparent by isotopic signatures of the methane gas. The higher *n*-alkane distributions of all mud volcanoes suggest an immature source rock and/or mixing with fresh organic matter confirming the observation made by the gas analyzes. As known from other studies, secondary microbial processes taking place in the associated petroleum reservoirs were confirmed by the isotopic signatures of CO_2. The observed positive isotopic signatures are typical for subsurface petroleum biodegradation followed by secondary methanogenesis. These processes can only proceed when water is available in the reservoirs. Beside the fact that the water provides microbial activities, the water composition itself was influenced by several secondary diagenetic processes such as illitization which proceed directly in the reservoirs e.g. depletion of sulfate caused by sulfate reduction or during the migration of the fluids, e.g. interaction and exchange with surrounding sediments and minerals, until they reach the water-mineral equilibrium. Further studies have to be focused on the deuterium and oxygen stable isotopic signatures of the water to exclude a meteoric water influence, on the lipid biomarker distribution of the fluids to differentiate between allochthonous and autochthonous signals and the associated processes inside the mud volcano system.

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Chapter 5

Geomicrobiology of fluid venting structures at the Salse di Nirano mud volcano area in the Northern Apennines (Italy)

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5.1 Introduction

Recent studies suggest that geological sources of methane like onshore and marine seeps, micro-seepage and mud volcanoes are an important source of this greenhouse gas (Etiope, 2004). They represent the second most important natural emission after wetlands. New estimations indicate a global emission of methane from mud volcanoes in a range of 6-9 Tg year-1 (Etiope and Ciccioli, 2009). Mud volcanoes are present in many terrestrial and marine areas worldwide (Mazurenko et al., 2003; Scholte, 2005), but their geographical distribution is strongly controlled by the geological settings. The majority is localized in areas of recent tectonic activity, particularly in zones of compression like accretionary complexes, thrust belts and e.g. in the forelands of Alpine orogenic structures (Dimitrov, 2002; Kopf, 2002). Furthermore, since the sediments in mud volcanoes are often hydrocarbon-rich, a relation between the formation of hydrocarbons and mud volcanoes was suggested (Dimitrov, 2002). The geographical occurrence of mud volcanoes in Italy can be divided into three main groups: (i) northern and (ii) central Italy and (iii) Sicily (Martinelli and Judd, 2004). The study site is located near Modena in Northern Italy, at a mud volcano area named Salse di Nirano. The Salse di Nirano is situated in the Emilia Romagna, covers a surface of approximately 75.000 m^2 and represents one of the biggest mud volcano areas in Italy (Martinelli and Judd 2004). Generally, mud volcanoes are formed by the expulsion of water, mud and gases (in particular methane and higher hydrocarbons), exhibit often anoxic niches and contain various electron acceptors. These preconditions potentially promote the activity of microorganisms performing the anaerobic oxidation of hydrocarbons, in particular methane. The anaerobic oxidation of methane (AOM) is suggested to be usually coupled to sulphate reduction (Reeburgh, 1980) and is carried out by a symbiotic association of methanotrophic archaea and sulphate reducing bacteria (SRB), namely members of the *Methanosarcinales* and the *Desulfosarcina/ Desulfococcus* group (DSS) (e.g., Hinrichs et al., 1999; Thiel et al., 1999; Boetius et al., 2000; Orphan et al,. 2001a, 2001b; 2002; Elvert et al., 2003; Reitner et al., 2005a, 2005b; Treude et al. 2005), although many aspect are still insufficiently understood. Recent phylogenetic and biochemical studies have suggested that the anaerobic methanotrophic (ANME) archaea have supposedly reversed the methanogenic

pathway (Hoehler et al., 1994; Hallam et al., 2003, 2004; Krüger et al., 2004). Although AOM has been mainly found in marine sediments and at marine methane seeps, AOM has been recently also found in a specific terrestrial mud volcano area near Paclele Micci in Romania (Alain, 2006). Our work aims to understand the functioning of the system of the Salse di Nirano mud volcanoes and the yet unknown microbial communities by organic-geochemical and molecular microbiological methods. The Salse di Nirano mud volcano area is located near Modena (Emilia Romagna, Northern Italy), and consists currently of four main conical vents and several satellite gryphons or “Salse”. The main volcanoes reach 3 m in height and emit muddy fluids and gas. The mud volcano area is situated at the bottom of an oval depression near an anticline in the outcrop of Plio-Pleistocene clays (Martinelli and Rabbi, 1998; Capozzi et al., 1994; Capozzi and Picotti, 2002; Accaino et al., 2007; Castaldini, 2008). The region belongs to an area of active thrusting along the Pede-Apennines margin of the Northern Apennines (Benedetti et al., 2003). Over the time the actual number and the shape of the cones varied (Martinelli and Judd, 2004). The surface colour of the mud volcanoes is grey. The emitted muddy fluids run down the cones; thereby, the clay deposits increase the size of the cones. During our observations in August 2008 and June 2009 several cones showed such an overflow, the other cones exhibited liquid mud breccias and gas bubbling deep in their craters. In the majority of the craters and smaller “Salse” gas bubbles from 1 cm up to 15 cm in diameter broken through the surface. At the surface of some mud pools an oily film of liquid hydrocarbons was visible. Here we describe the environmental conditions and our first results of the geochemical and biochemical analyses of the phases seeping out of the at this exceptional geomicrobiological environment.

5.2 Materials and Methods

5.2.1 Study site and sampling

The samples were collected during two campaigns (August 2008 and June 2009) from several mud volcanoes in the Emilia Romagna area in Northern Italy. Mud and gases were collected from four active cones, which showed constant development of gas bubbling. This study is focused on one of the active cones of the Salse di Nirano (Fig. 24). Mud samples were collected in a depth of approximately 1.5 m with a hand-operated vacuum pump, connected to a flexible

tube with a metal pipe at the open end (UniSampler, Bürkle, Bad Bellingen, Germany) and were transferred to the laboratory under cooled conditions. The samples for the lipid analyzes were freeze-dried and stored by -20°C. Gas bubbles were collected in the central part of the gryphon using a funnel and special gas vials (Labco Vials, Labco Limited, Buckinghamshire, United Kingdom). The temperature, redox potential and the pH-values of the mud were measured directly in the pools.

5.2.2 Gas analysis

The concentrations of CH_4, CO_2 and N_2O were measured with an automated gas chromatographic system (GC 14A, Shimadzu, Duisburg, Germany) equipped with two detectors, a flame ionization detector (FID) for CH_4 analysis and an electron capture detector (ECD) for CO_2 and N_2O analyses (detailed description: LOFTFIELD, 1997).The carbon isotope compositions ($\delta^{13}C$, $\delta^{2}H$) of the methane were measured with an isotope ratio mass spectrometer (IRMS; Delta plus XP, Thermo, Bremen, Germany; $\delta^{13}C$) coupled to a PreCon and a High Temperature Conversion / Elemental Analyzer in conjunction with an IRMS (Delta V plus, Thermo, Bremen, Germany; $\delta^{2}H$) .

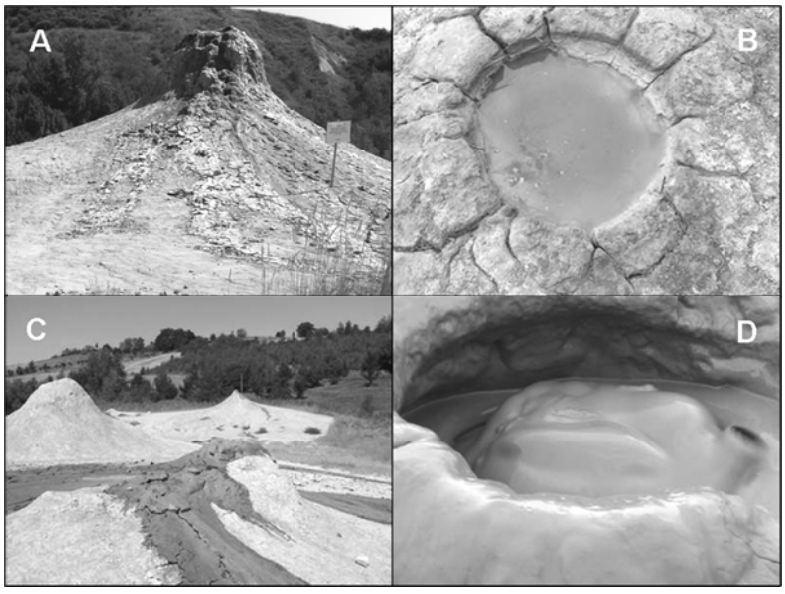

Figure 24: Images of the Salse di Nirano mud volcanoes Northern Apennines, Italy. A) and C) mud volcano cones and craters of the Salse di Nirano. B) Liquid hydrocarbons on the surface of a pool. D) A bubbling salse.

5.2.3 Geochemical analyses

The total carbon (TC), nitrogen (TN) and sulphur (TS) concentrations were measured by a CNS Elemental analyser (Euro Vector Instruments and Software, Milano, Italy). The total inorganic carbon (TIC) was determined after acidification with H_3PO_4. The total organic carbon (TOC) was calculated by the difference between TC and TIC. The elemental content of the particle-free fluids were analyzed by an Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES; Perkin Elmer Optima 3300 DV) according to Thomson and Walsh (1983).

5.2.4 Lipid biomarker analyses

Lyophilised and homogenised samples (about 4 g dry weight of campaign 2008 and about 30 g of campaign 2009 sample, respectively) were hydrolysed using 6 % KOH in methanol (pH 14) in excess (2h at 80°C in ultrasonication bath) to extract free and release ester-bound lipids. The resulting alkaline reaction solution was extracted with *n*-hexane (5x) yielding the neutral lipids fraction. The neutral lipid fraction was further separated by column chromatography (Merck silica gel 60) and eluents of increasing polarity (n-hexane, dichloromethane, MeOH) providing a hydrocarbon and a polar fraction containing mainly alcohols. The alcohol fraction was treated with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for 2 h at 80°C to silylate alcohols. Fatty acids (FA) were obtained by acidification of the residue of the alkaline reaction solution to a pH of 1-2 and subsequently extracted using *n*-hexane (5x). Prior to analysis, fatty acids were converted to their methyl esters by adding trimethylchlorosilane in methanol (1:9; v:v; 2 h, 80°C). The above mentioned fractions were analyzed by gas chromatography-mass spectrometry (GC-MS) using a Varian 1200L MS (EI, 70 eV) coupled to a Varian CP 3800 GC equipped with a fused silica capillary column (ZB5-MS, 30 m, 0.32 mm inner diameter, 0.25 µm film thickness). Carrier gas was Helium. Temperature program: 3 min at 80°C; from 80°C to 310°C at 6°C min^{-1}; 20 min at 310°C. Lipids were identified by comparison of GC-retention times and published mass spectra of reference compounds (NIST library and own spectra). $\delta^{13}C$-values of lipids were analyzed (min. of three replicates) using a Thermo Trace GC coupled to a Thermo Delta Plus isotope-ratio mass spectrometer (GC-C-IRMS). Combustion of the components to CO_2 was performed with a CuO/Ni/Pt-furnace operated at 940°C. The stable carbon isotope compositions are reported in the delta notation ($\delta^{13}C$)

vs. the V-PDB standard (standard deviation was usually less than 0.5 ‰). The typical GC-program used for GC-C-IRMS analyses was the same as described above for GC-MS.

5.2.5 Microbiological methods

Cultures for isolation of sulphate reducing bacteria were prepared anaerobically. Media were composed as already described, with lactate or ethanol as carbon sources (Postgate, 1951; Sakaguchi et al., 2002). Liquid media were inoculated with different volumes (10 % - 50 %) of samples from mud volcano fluids. From enrichment cultures, grown at 20°C, pure cultures were obtained after separation of colonies by agar tube dilution series (Evans et al., 1977). The strains were further characterised by 16S rDNA-analysis according to established procedures (primers: 27F, 1525R, Lane; 1991, E334F, E939R, Baker et al., 2003). Negative staining of cells was performed with 1% phosphotungstic acid (Hoppert, 2003). Electron microscopy was performed with a Zeiss EM 902 transmission electron microscope.

5.3 Results and discussion

5.3.1 Geochemistry

The largest part of the collected gas emitted by the mud volcanoes at the Salse di Nirano was methane (~ 99%), but small amounts of other hydrocarbons, carbon dioxide and nitrous gas were also found to be present (< 1%). Measurements of the $\delta^{13}C$-values of the released CH_4 gave a mean value of –50 ‰, and a $\delta^{2}H$ -value of -175‰. The $\delta^{13}C$-value indicates a thermogenic origin of the gas, potentially with minor contributions from a biogenic source (Whiticar, 1999). The material expelled is semi-liquid and is composed of 24% carbonates, 34% feldspar, 41% quartz and 1% halite analyzed by XRD (X-Ray diffraction). The content of the total inorganic carbon (TIC) in the mud is 2.64 % dry weight and the total organic carbon is 0.41% dry weight (Table 10). The amount of total sulphur is 0.19% dry weight and for the total nitrogen the analyses revealed a content of 0.04% dry weight.

As became evident during the lipid biomarker analyses the mud samples contain high amounts of liquid hydrocarbons. The hydrocarbon fractions of both

campaigns were dominated by oil hydrocarbons with carbon chains from C_{18} to C_{38}, with a maximum at C_{28} and no odd over even predominance. This distribution clearly shows the presence of mature organic matter in the underlying sediments of the mud volcanoes at the Salse di Nirano. A similar predominance of *n*-alkanes was also found in mud from Romanian mud volcanoes (ALAIN et al., 2006). Furthermore, fresh allochthonous organic matter, mainly from higher plants, was also identified in the polar fraction and may be transported into the mud via wind and/or by the flow of the mud through the underlying palaeosoils. These components mainly include even-chained wax ester-derived homologues of long-chain fatty acids and long chain alcohols, both maximizing at C_{26} ($\delta^{13}C$ about -30 to -33‰) and suite of plant derived sterols (e.g. Stigmastanol, Fig. 26). A low redox potential of -110 mV, a pH-value of 7.9 and a temperature of about 16°C was measured for the mud pool analysed for this study (Table 1). Elemental analyses of the particle-free fluid water revealed a concentration of 191 ppm Mg^{2+}, 81 ppm Ca^{2+} and 4492 ppm Na^{+}. These data evidenced that the waters are brackish and are similar to spring and groundwaters from the same area (Conti, 2000). The low redox potential, the pH-value, the temperature of about 23°C and the presence of various electron acceptors (e.g. SO_4^{2-} from underlying gypsum layers) allow microbial sulphate reduction and establishment of diverse sulphate reducing bacteria (SRB) as well as the growth of other microorganisms.

Table 10: Geochemical composition and environmental conditions of a Salse di Nirano mud volano (A: pore water: B: mud)

A		[mV]		[°C]				[ppm]			
	Sample	E_H (corr)	pH	T	Mg^{2+}	Ca^{2+}	Fe^{2+}	Ni^{2+}	SO_4^{2-}	Cl^-	Na^+
	NR7	-110	7.9	23	191	81	0.1	0.04	200*	7300*	4492
B		% [w/w]									
	Sample	TIC	TOC	TS	TN						
	NR7	2.49	0.43	0.19	0.04						

* from: Martinelli and Ferrari (1991)

5.3.2 Microbiology - Enrichment cultures

Conditions for enrichment of organisms aimed at the isolation of sulphate-reducing bacteria. The samples were taken from two mud volcanoes. In the original mud volcano fluids, occasionally, large vibroid cells could be observed. From the enrichment cultures, four different pure cultures of anaerobic bacteria were obtained. 16S rDNA analysis revealed 99 – 100 % identity of the isolates to already described organisms: The isolates include the sulphate-reducing Gram-negative *Desulfovibrio psychrotolerans*, which was, so far, only known from a salt water lake situated in the Himalaya (Jyothsna et al., 2008). It may be due to the presence of yeast extract in the enrichment media that also (facultative) fermenting organisms were isolated. In the mud volcano fluid, the *Clostridium thiosulfatireducen*-isolate may ferment proteins from other bacterial sources. The organisms are depicted in Fig. 25. The flagellated *Clostridium* cell exhibits a terminal spore (Fig. 25a), *Desulfovibrio* has a vibroid cell shape and shows one polar flagellum (Fig. 25b). The occurrence of two bacteria from mammalian intestines (*Enterobacter aerogenes* and *Enterococcus faecalis*) may be explained by contamination of the setting with ground- or surface water from pastures situated around the sampling site. From the enrichment cultures and isolates as well as the outgasing of H_2S at the sampling site it is obvious that sulphate reduction in the fluids is an important process. Archaea have, so far, not been isolated from the mud.

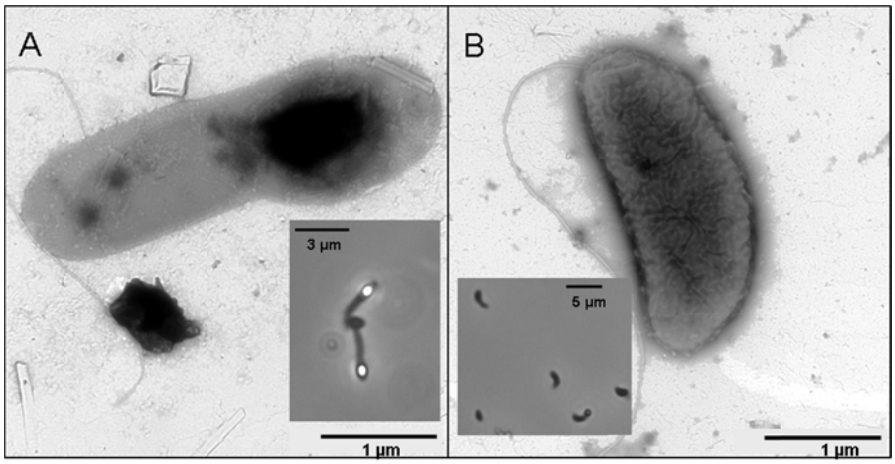

Figure 25: Electron micrographs of the isolates *Clostridium thiosulfatireducens* (A) and *Desulfovibrio psychrotolerans* (B)-strains after negative staining. The insets show light micrographs of the respective strains

5.3.3 Lipid Biomarkers

Eukaryotes, bacteria, and archaea are characterised by partially very specific lipid membrane components, so called lipid biomarkers. Moreover, ratios of stable carbon isotopes in these biomarkers contain information on the carbon fixation pathway used, the biosynthesis of lipids but especially on the carbon substrate used (Hayes 2001). In particular, processes where ^{13}C-depleted substrates are used by microorganisms can be easily identified by compound specific stable isotope analyses even in complex and heterogeneous microbial settings. An excellent example is the anaerobic oxidation of methane (AOM), where the usually isotopically light methane carbon is transferred into the lipids of the closely operating AOM performing consortia of sulphate-reducing bacteria and methane oxidizing archaea (e.g., Blumenberg et al., 2004; Hinrichs et al., 1999; Pancost et al., 2001b). In addition to the above mentioned allochthonous biosignatures, we also found biomarkers with slightly ^{13}C-depleted stable isotope ratios suggesting microbial sources which fed on ^{13}C-depleted carbon substrates. These structures are several dialkyl glycerol diethers (DAGE) with the highest depletion of -51.7‰ (campaign 2008) found for a DAGE with two hexadecane chains (16/16-DAGE). Also depleted are *ai*15/*i*15-DAGE (-41.4‰) and the *ai*15/*ai*15-DAGE (-50.1‰) with the first suggesting contributions of sources with partially conventional δ^{13}C values. Respective DAGE have so far mainly described from thermophilic bacteria like *Aquifex pyrophilus* (Huber et al., 1992) and the sulphate-reducing bacterium *Thermodesulfobacterium* commune (Langworthy et al., 1983). However, at many marine, normal temperated sites where the AOM is the predominant microbial process, strongly ^{13}C-depleted DAGE occur in high amounts. Consequently, these DAGE were interpreted as been sourced by AOM-involved SRB (Blumenberg et al., 2004; Elvert et al., 2005; Pancost et al., 2001a). We therefore interpret a high proportion of *i*15/*ai*15-, *ai*15/*ai*15- and 16/16-DAGE present in the mud of the Salse di Nirano to be produced by SRB involved in AOM. However, since the δ^{13}C of the methane was found to be -50‰ and that AOM-performing microbes further fractionate the isotopic signal of the methane towards more negative values a high proportion of the tentatively AOM SRB-derived DAGE must be also sourced by additional SRB or other bacteria. For instance, glycerol ether lipids with *iso*-branched pentadecane chains were recently also found in the spore-forming and

widespread soil bacterium Myxobacterium *Myxococcus xanthus* (RING et al., 2006) which might be also partial sources of *i*15/*i*15-DAGE in the mud, explaining the mixed $\delta^{13}C$ signal found in the mud.

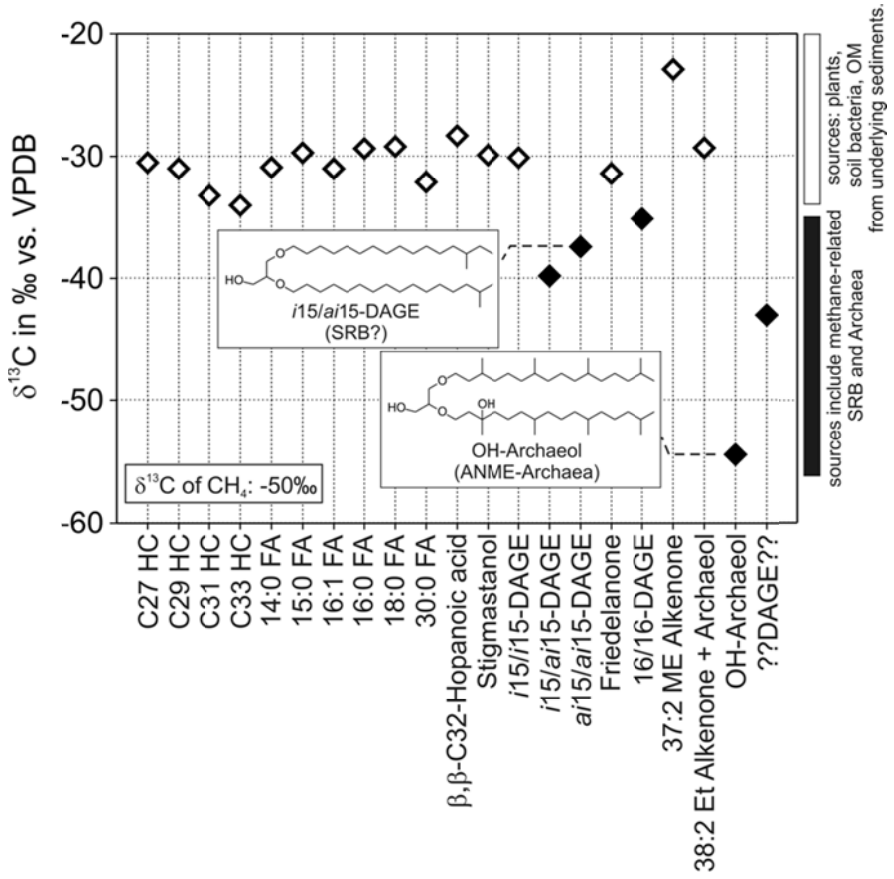

Figure 26: Stable carbon isotopes of selected lipid biomarkers extracted from mud samples from the 2009 campaign and the potential sources (exemplified two structures). Note that the amount of OH-archaeol was very low. Abbreviations: HC = hydrocarbon; FA = fatty acid; DAGE = dialkyl glycerol ether; SRB = sulphate-reducing bacteria; ANME = anaerobic methanotrophic archaea.

The majority of archaeal biomarkers present in the mud are only slightly or not ^{13}C-depleted (particularly archaeol from the 2008 campaign -36 and from the 2009 campaign -29.3‰ although in the latter sample co-elution with a C38:2 ethyl alkenone was observed). Isoprenoid hydrocarbons such as crocetane and 2,6,10,15,19-pentamethylicosane, all often present at AOM-sites (e.g. Michaelis et al., 2002; Thiel et al., 1999), are not present or, more likely, are superimposed by other co-eluting components. Nevertheless, the vast majority of archaea present in the mud is obviously neither performing the anaerobic oxidation of methane nor feeding on any other ^{13}C-depleted carbon source. Nevertheless, the presence of trace amounts of hydroxyarchaeol (OH-archaeol) with a $\delta^{13}C$ value of -51‰ demonstrates that archaea are also present which are actively involved in the

anaerobic turnover of methane.We also observed differences in the distribution and stable isotope signatures of biomarkers tentatively sourced by microorganisms performing the anaerobic oxidation of methane, between both sampling campaigns. Higher concentrations, accompanied by stronger ^{13}C-depletions were found in the sample from the 2008 campaign (strongest ^{13}C-depletion -51.4‰ for 16/16-DAGE) whereas the same compound in the mud sample from the 2009 campaign revealed a $\delta^{13}C$ value of -41‰ (Fig. 26). This demonstrates that the proportion of AOM-involved bacteria and archaea was different at both sampling times, suggesting the intensity of AOM to vary strongly with respect to time and/or space.

5.4 Conclusions

Terrestrial mud volcanoes of the Salse di Nirano were found to be excellent settings for divers microorganisms. The bacterial lipid biomarkers and the enrichment cultures support the presence of sulphate reducing bacteria in the mud volcanoes. Furthermore, lipid biomarkers demonstrate that methanotrophic archaea are present in the system of the Salse di Nirano mud volcanoes suggesting that AOM is taking place. The slightly ^{13}C-depleted stable isotope ratios of the bacterial biomarkers indicates that these microorganism feed on ^{13}C depleted carbon sources. However, the low content of the archaeal biomarkers in the samples like archaeol and hydroxyarchaeol and the fact that these biomarkers are only slightly or not depleted in ^{13}C shows that AOM in this fluid venting structures it is not very important *in situ*. Our data indicate that the majority of the yet unknown archaea and bacteria present in the mud are neither involved in AOM nor feeds on any other ^{13}C-depleted carbon source.

Acknowledgments

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Chapter 6

Terrestrial mud volcanoes of the Salse di Nirano (Italy) as a window into deeply buried organic-rich shales of Plio-Pleistocene age

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Abstract

The terrestrial mud volcanoes of the Salse di Nirano are situated in the Northern Apennines (Italy) and were formed by the expulsion of mud, water, liquid hydrocarbons and gases, predominantly methane (CH_4). Previous studies revealed that methane is consumed by microbial associations using the anaerobic oxidation of methane (AOM), a process also occurring in marine settings. This was supported by the presence of ^{13}C-depleted biomarkers specific to sulfate-reducing bacteria and archaea. However, the vast majority of biomarkers appeared to have other, so far unexplained sources, than microorganisms that in situ feed on hydrocarbon gases. Therefore, lipid biomarker distributions of fluid samples from the Salse di Nirano mud volcanoes were revisited and compared to those extracted from organic-rich shales from the underlying geological formations. The organic chemical analyses of the mud volcano fluids revealed signals of various eukaryotic, bacterial and archaeal organisms. In addition to signals from higher plants, specific bacterial dialkyl glycerol diethers (DAGE; in particular *ai*15/*ai*15 and 16/16) were found, which putatively originate from sulfate-reducing bacteria (SRB). The presence of archaea is evidenced by archaeol and trace amounts of sn2-hydroxyarchaeol. Most biomarkers were not depleted in ^{13}C, suggesting mainly non-methane-consuming source organisms. Organic chemical analyses of the Plio-Pleistocene shales from the underlying geological formation revealed the same pattern for most of the bacterial and archaeal components. The strong similarities between both samples suggest that the majority of the biomarkers in the emitted fluids in Nirano originate from these marine, organic-rich deposits through which the fluids passed. These biomarkers clearly obscure signals from microorganisms growing in situ in the mud volcanoes, but the extent of this process is spatially and temporarily highly variable.

Keywords Terrestrial mud volcano, Northern Italy, Lipid biomarkers, Shales, mud fluids

6.1 Introduction

Mud volcanoes are geological structures formed by the emission of argillaceous material, water, brine, gas and oil (Milkov, 2000). Such fluid venting structures occur in terrestrial and marine environments worldwide and are caused by tectonic stress (Brown, 1990; Dimitrov, 2002; Kopf et al., 2001, 2002; Milkov, 2000; Niemann and Boetius, 2010). These processes can lead to over-pressured pore fluids and instable sediments and, finally, to emissions of mud, water and gas. In the Northern Apennine chain, mud volcanoes occur along the external compressional margin, mostly located in the Emilia-Romagna region (Conti et al., 2003). A case in point is the Natural Reserve of “Salse di Nirano” near Modena (Fig. 27c), one of the largest mud volcano areas in Italy (Martinelli and Judd 2004). The Nirano mud volcanoes periodically emit semi-liquid material (hereafter referred to as fluid) and gas. While gas emissions mainly consist of methane (~99%) and C_2 to C_4 hydrocarbons (<1%; Heller et al., 2011), the fluids consist of a muddy matrix and rock clasts with diameters of a few mm (Accaino et al., 2007). These fluids passed through different geological formations from the Jurassic to the lower Pleistocene (Bonini et al. 2008 and references therein; Fig. 28). Mud fluids collected at the Salse di Nirano contain various electron acceptors for microbial organic matter turnover (e.g., SO_4^{2-}; Heller et al., 2011). Although concentrations might vary considerably, preconditions potentially promote the activity of microorganisms performing aerobic and the anaerobic oxidation of hydrocarbons, in particular, methane (de Beer et al., 2006; Nauhaus et al., 2007; Wrede et al. (this issue)). The latter process, namely anaerobic oxidation of methane (AOM), is often found to be coupled to sulfate reduction (Reeburgh, 1980), although recent studies have also shown the capability of certain microorganisms to anaerobically oxidize methane with electron acceptors other than SO_4^{2-} (Raghoebarsing et al., 2006, Ettwig et al., 2008, Beal et al., 2009). The importance of these alternative processes in terrestrial and aquatic settings, however, is still unknown. The sulfate-dependent AOM is carried out by a consortium of methanotrophic archaea and sulfate reducing bacteria (SRB) (e.g., Hinrichs et al., 1999; Boetius et al., 2000; Orphan et al., 2001a, 2001b; 2002; Reitner et al., 2005a, b). Although sulfate-dependent AOM mostly occurs in marine

environments, evidence for this process in a terrestrial setting has been described for mud volcanoes near Paclele Mici in Romania (Alain et al., 2006).

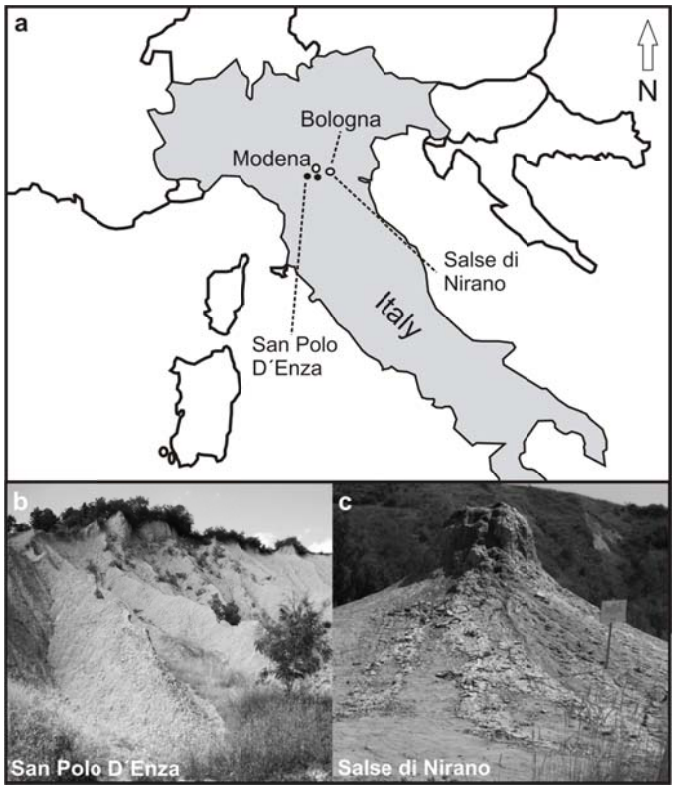

Figure 27: a) Location of study sites in the Northern Apennines (Italy). b) Plio-Pleistocene sediments of San Polo D´Enza. c). Salse di Nirano mud volcano NR7.

Structurally specific lipids are often useful indicators of their respective source organisms (biomarkers) and, moreover, they are one of the major organic carbon pools in all living organisms. Furthermore, ratios of stable carbon isotopes in these biomarkers contain information on the carbon fixation pathway, the biosynthesis of lipids and, especially, the carbon substrate used (Hayes, 2001).

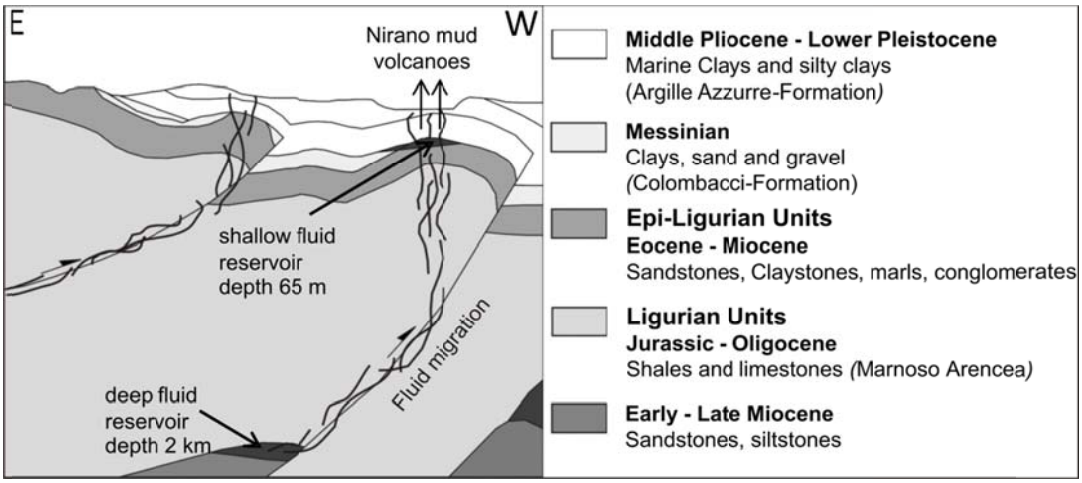

Figure 28: Geological setting of the Natural reserve Salse di Nirano and the Argille Azzurre shales in the Northern Apennines (Italy; adapted from Bonini 2008).

To obtain deeper insight into the system of the fluid venting structures of the Salse di Nirano, we analyzed the lipid biomarker composition of a mud volcano of the Salse di Nirano. An overview of the results of biomarker extractions of mud sampled during two campaigns (2008 and 2009) has been previously published (Heller et al., 2011). Major findings from that study were that the fluids sampled in 2008 contained mostly allochthonous organic matter and, to a lesser extent, biomarkers with slightly ^{13}C-depleted stable isotope ratios, suggesting microbial sources fed on ^{13}C-depleted carbon sources. These structures consisted of several dialkyl glycerol diethers (DAGE; 16/16-DAGE (-51.7‰), *ai*15/*ai*15-DAGE (-50.1‰)). The archaeal biomarker archaeol was found to be only slightly ^{13}C-depleted (-36‰). Nevertheless, the fluids also contained small amounts of sn-2-hydroxyarchaeol with a δ^{13}C-value of -51‰ demonstrating that archaea are also present that are actively involved in the anaerobic turnover of methane. The Salse di Nirano mud volcano fluids sampled in 2009 showed a slightly different distribution of the lipid biomarkers (Heller et al., 2011). The fluids also contained DAGE in a similar pattern to that found in the mud sampled in 2008, although ^{13}C-depletions were generally less pronounced (e.g., *ai*15/*ai*15-DAGE -35.3‰). In the mud collected in 2009, archaeol (δ^{13}C-value = -29.3‰) and trace amounts of ^{13}C-depleted sn-2-hydroxyarchaeol (-54‰) were also observed (Heller et al., 2011). In contrast to the mud sampled in 2008, the 2009 campaign revealed high amounts of the long-chained alkenones heptatriacontadien-2-one (37:2one) and

octatriacontadien-2-one (38:2one) with the latter co-eluting with archaeol. 4,23,24-trimethylcholest-22-enol (dinosterol) was found to be highly abundant in both samples (Heller et al., 2011). Because some of the biomarkers were highly abundant, particularly those of sulfate-reducing bacteria, the low ^{13}C depletion seen in most biomarkers is evidence that AOM in this fluid venting structure is generally not very important, and that AOM and other in situ microbial processes are, to varying extents, obscured by allochthonous organic matter (Heller et al., 2011). In addition to recent or sub-recent microbial processes as sources for the organic matter in the mud fluids, other origins were suggested; however, likely sources were not further studied (Heller et al., 2011). Potential origins include deep geological formations, because, in Nirano, the fluids pass through several organic rich sedimentary rocks, particularly from the late Pliocene (Bonini et al., 2008).

Here we describe new data on the composition of biomarkers of a prominent underlying organic-rich shale (Fig.27b) deposited during the late Pliocene to early Pleistocene. For comparison, previously studied hydrocarbon and biomarker patterns of mud fluids of the Salse di Nirano sampled in 2009 (Heller et al., 2011) are revisited and are presented in more detail to characterize the impact of organic matter from geological formations on the patterns in the mud volcano (Heller et al., 2011; Fig.27c).

6.2 Geological Setting

In Italy, mud volcanoes occur along the external compressional margin of the Apennine chain (Pellegrini et al., 1982, Capozzi et al,. 1994). There, the fluid venting structures are distributed along two belts (Borgia et al., 1986, Minissale et al., 2000). The first belt stretches along the Po Plain foothills, while the second is more internal and runs nearly parallel to the main apenninic divide (Capozzi and Picotti, 2002). The Nirano mud volcano field belongs to the seeps that occur in the Po plain foothills. These chains and their complex structures are the result of the collision between the European and the Adriatic plates from the Mesozoic to the present when the Adriatic plate acts as a promontory of the African plate (Accaino et al. 2007 and references therein). The geological formations that were formed during this process are the Ligurian unit, the sub-Ligurian units, the Tuscan nappe

and the Cervarola unit, the Umbria-Romagna and Marche-Adriatic thrust units and the Epi-Ligurian sequences (Ricci Lucchi, 1986; Accaino et al., 2007). The Ligurian unit that forms the upper tectonic nappe of the Apenninic chain consists of ophiolites and oceanic sediments (Jurassic to Eocene age). From the late Eocene until the Plio-Pleistocene, the Ligurian unit migrated northeastward. During this Tertiary translation, Epi-Ligurian units consisting of marine sediments were deposited above the Ligurian unit in satellite basins on the top of the migrating frontal thrust (Ricchi Lucchi, 1986; Vai and Martini, 2001). In the Neogene, the foreland basin migrated to the northeast, coupled with the progressive accretion of the thrust wedge (Ricci Lucchi, 1986).In the Northern Apennines, the Ligurian unit overlays the Messinian evaporites (gypsum) and the Miocene turbidites of the foredeep, which overlie the Mesozoic-Palaeogene carbonates. From the late Messinian to the early Pliocene, thrusting telescoped the entire structure of the chain (Pieri and Groppi, 1981; Castellarin et al., 1986). Until the early middle Pliocene, the foredeep succession was affected by thrusting and erosion. During the late Pliocene, these activities stopped and organic-rich sediments (Upper Pliocene to Pleistocene) onlapped and covered the ramp. After this time a system of normal faults occurred (NW-SE-trending, SW-dipping, connected with SW-NE-oriented faults) (Accaino et al., 2007). At present, the Salse di Nirano mud volcano field is located in an oval depression and covers a surface of approximately 75.000 square meters (Martinelli and Judd, 2004). The elliptical depression is the result of a collapse of a “mud diapir” at the end of the uplifting activity in the area. The mud volcano area is connected with an anti-apenninic fault line cutting a little anticline (Bonini 2007, 2008) and deforming the Plio-Pleistocene argillaceous sediments (Fig. 28). The fault, which is related to Nirano, is probably linked to the Sassuolo fault scarp line. This line is located two km north of the Nirano valley and represents the main pathways of fluid expulsion (Accaino et al. 2007). Currently, the Nirano mud volcano area is formed by four main vents associated with some smaller active pools. The emitted mud breccias of the Nirano mud volcanoes consist of submillimeter angular fragments (Accaino et al., 2007) of claystones and carbonates belonging to the Argille Azzurre Formation (Plio-Pleistocene) and to the underlying Eocene-Miocene Epi-Ligurian and Jurassic Ligurian units (Fig. 28; Bonini et al., 2008).

6.3 Materials and Methods

6.3.1 Study site and sampling

The shale sample was collected in June 2009 from the Plio-Pleistocene Argille Azzurre Formation near San Polo D´Enza (N44°37'30.72", E10°26'54.72", Fig. 27b). Sampling at Nirano was conducted in March 2008 and June 2009 (N44°30'45.54", E10°49'17.4", Fig. 27c). Selected data of the Nirano samples, particularly compound specific isotope signatures of biomarkers without further details on the distribution, were already published in Heller et al. (2011).

6.3.2 Geochemical analyses

The total carbon (TC), nitrogen (TN) and sulfur (TS) concentrations were measured by a CNS elemental analyzer (Euro Vector Instruments and Software, Milano, Italy). The total inorganic carbon was determined after acidification with H3PO4 and was also determined on a CNS elemental analyzer (Euro Vector Instruments and Software, Milano, Italy). The total organic carbon (TOC) was calculated by the difference between TC and TIC. Mineral phase analyses of the freeze-dried fluids were performed on a Philips X Pert MPD (X-Ray-diffraction) equipped with a PW3050 Goniometer (Cu as anode material). Data were collected from 4 to 65°2θ using a step size of 0.02°2θ and a count time of at least 2 seconds per step. The sulfate concentration was determined on an Ion chromatography with chemical suppression and conductivity detection (Metrohm).

6.3.3 Lipid biomarker analyses

A total of 100 g of homogenized shale were hydrolyzed using 6 % KOH in methanol (pH 14) in excess (2h at 80°C in ultrasonication bath), in order to release ester-bound lipids and to extract free lipids. The resulting alkaline reaction solution was extracted with n-hexane (5x), yielding the neutral lipid fraction. This fraction was further separated by column chromatography (Merck silica gel 60) and eluents of increasing polarity (n-hexane, dichloromethane, methanol), providing a hydrocarbon fraction and a polar fraction containing mainly alcohols and ketones. The polar fraction was treated with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for 2 h at 80°C to silylate alcohols. Fatty acids (FA) were obtained by acidification of the residue of the alkaline reaction solution to a pH of 1-2 and

subsequently extracted using n-hexane (5x). Prior to analysis, fatty acids were converted to their methyl esters by adding trimethylchlorosilane in methanol (1:9; v:v; 2 h, 80°C) and extraction with n-hexane. The above-mentioned fractions were analyzed by gas chromatography-mass spectrometry (GC-MS) using a Varian 1200L MS (EI, 70 eV) coupled to a Varian CP 3800 GC equipped with a fused silica capillary column (Phenomenex ZB5-MS, 30 m, 0.32 mm inner diameter, 0.25 µm film thickness). The δ13C-values of lipids were determined (minimum of three replicates) using a Thermo Trace GC coupled to a Thermo Delta Plus IRMS (GC-C-IRMS). Combustion of the components to CO_2 was performed with a CuO/Ni/Pt-furnace operated at 940°C. Stable carbon isotope compositions are reported in delta notation ($\delta^{13}C$) in reference to the V-PDB standard (standard deviation was usually less than 0.5 ‰). For more details, see Heller et al. (2011).

6.4 Results

6.4 1 Bulk composition

The X-ray-diffraction (XRD) analyzes revealed quartz as the dominant phase in the San Polo D´Enza shale, the main carbonate phase is calcite, whereas the accessory minerals are albite, chlorite and mica. The content of total inorganic carbon in the shales was 2.5% and the total organic carbon was close to 0.5% (Table 11). The total nitrogen (TN) content was 0.06%, and the total sulfur (TS) content was also 0.06%. The composition of the dried fluid of the mud volcano was published by Heller et al. 2011 (see also Table 11). The sulfate concentration in the emitted fluids of the Salse di Nirano mud volcanoes ranged from 8.0 up to 193 mg l-1 (campaign 2008).

6.4.2 Higher hydrocarbons (C_{15+})

Figure 29 shows the hydrocarbons in the shale from the Plio-Pleistocene Argille Azzurre Formation (carbon chains C_{17} to C_{40} with a maximum at C_{29}/C_{31}). The hydrocarbon pattern demonstrates the highest abundances of long chained compounds and a strong odd-over-even predominance in the higher molecular weight range. In the lower hydrocarbon range, a modal distribution was observed with a maximum at about C_{22}.

Table 11 Organic and inorganic components of the dried mud volcano sample and the Plio-Pleistocene shale of San Polo D´Enza (% w/w = percent by weight).

Elemental parameter	Plio-Pleistocene shale (BS-09) (%w/w)	Salse di Nirano mud volcano (NR7-09) (% w/w)
Total inorganic carbon	2.50	2.55
Total organic carbon	0.46	0.42
Total nitrogen	0.06	0.05
Total sulfur	0.06	0.17

6.4.3 Lipid biomarkers

The distribution of biomarkers in the polar fraction obtained from the organic-rich shale (BS-09) after alkaline hydrolysis is shown in Figure 4(b/d). $\delta^{13}C$ values of selected peaks are shown in Table 12. 4-desmethyl stanols and stenols ($C27\Delta^0$, $C_{28}\Delta^0$, $C_{29}\Delta0$ and $C_{29}\Delta^5$) and long chain n-alcohols with a strong even-over-odd predominance were the major compounds in the polar fraction of the shale from San Polo D´Enza (Fig. 30b). Dialkyl glycerol diethers (DAGE) were also considerable constituents of the polar fraction of the shale. In Figure 4d, the ion trace m/z 130 highlights the respective distribution. Highest amounts were found for a DAGE with two hexadecane chains (16/16-DAGE) and three with terminally branched pentadecane chains (*i*15/*i*15-DAGE, *ai*15/*i*15-DAGE, and ai15/ai15-DAGE). Fatty acids (carbon chains between C_{14} and C_{32}) were also analyzed, but are not shown. Among those, highest abundances were observed for long chain structures (carbon chains of C_{20}-C_{32}) with an even-over-odd predominance. However, considerable amounts of short chain fatty acids, including monounsaturated (e.g., hexadecenoic acid) and terminally branched structures (e.g., ai15FA; ai-pentadecanoic acid) were also found. Selected $\delta^{13}C$-values of fatty acids are shown in Table 2. The shale also contained small amounts of archaeol which was not depleted in ^{13}C (Table 12). Isoprenoid hydrocarbons such as crocetane and 2,6,10,15,19-pentamethylicosane were not found. Heptatriacontadien-2-one (37:2one) and octatriacontadien-2-one (38:2one) were also observed in considerable amounts, with the latter co-eluting with archaeol. 4,23,24-trimethylcholest-22E en-3β-ol (dinosterol) was also highly abundant.

6.5 Discussion

6.5.1 General

Mud volcanism in Northern Italy is caused by tectonic compression events, and results in the extrusion of mud fluids and gas bubbles. In Nirano, the gas is mainly composed of methane (~99 %; $\delta^{13}C$ = -50‰ VPDB, $\delta^{2}H$ = -175‰ SMOW; Heller et al. (2011)), with only trace amounts of CO_2, N_2 and higher hydrocarbons. The fluids arise from a deep fluid reservoir located at a depth of 2 km in the Ligurian units. Moreover, on their way up, the fluids reach another shallow reservoir at 60 m depth, at the boundary between Miocene and Plio-Pleistocene depositions (Bonini et al. 2008 and references therein). From there, the fluids rise to the surface. The total organic carbon of the emitted fluids was close to 0.4% (percent by weight; Heller et al. 2011), which is nearly the same as the total organic carbon content of the organic-rich shales of the Plio-Pleistocene at 0.5% (percent by weight; Table 11). Total nitrogen, total inorganic carbon and total sulfur also demonstrate similar abundances, suggesting a close geological relationship between both samples.

6.5.2 Allochthonous biomarkers versus biomarkers of recent microbial methane turnover

Fluids from terrestrial mud volcanoes harbor diverse microorganisms, capable of using the reduced, thermogenically-produced hydrocarbons as substrates (Alain et al. 2006; Heller et al. 2011). This includes methane, which can be oxidized aerobically and anaerobically. Previous studies on mud volcanoes of the Salse di Nirano contained, although low in concentration, biomarkers specific to in situ microbial turnover. However, the majority of biomarkers in the fluids, particularly those extracted from the 2009 sample, were most likely allochthonous in origin; the distinct sources were still unclear (Heller et al. 2011). To better understand the sources of organic matter, we studied the underlying formations that the fluids pass by and compared these results with those from the fluids of the mud volcano NR7 published in Heller et al. 2011. We also revisited the hydrocarbon and biomarker distributions from that study, and present them in more detail than in our previous publication.

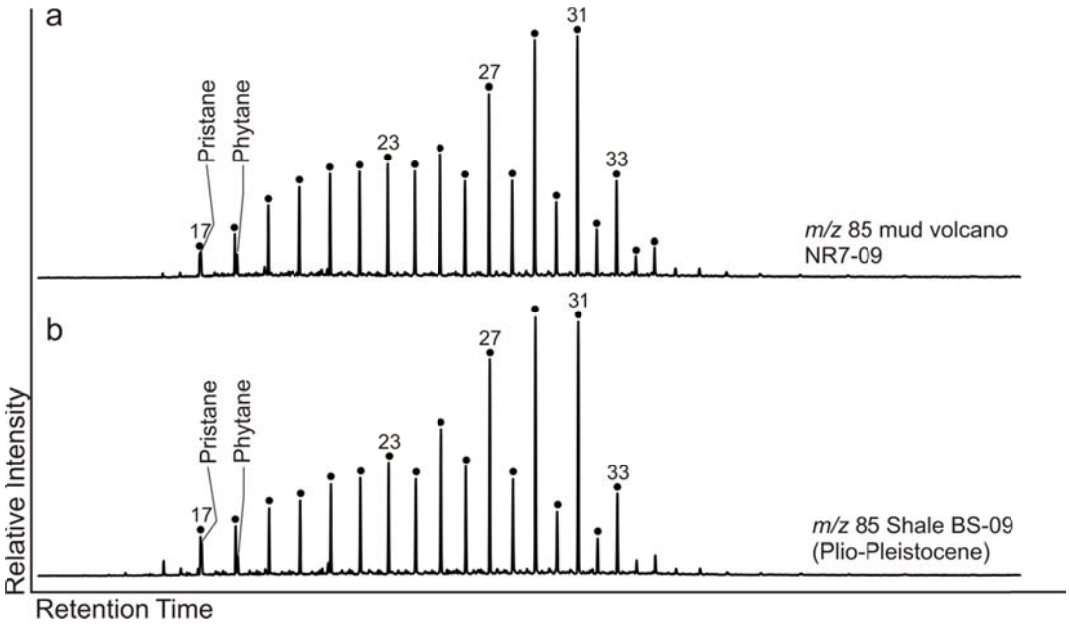

Figure 29: Gas chromatograms (traces of ions with m/z 85) depicting the distribution of n-alkanes extracted from a) the Salse di Nirano mud volcano NR7-09 and b) the organic-rich shales (BS-09) of San Polo D´Enza. Dots/numbers indicate carbon chain lengths of n-alkanes.

Hydrocarbons from the shales of the Plio-Pleistocene Argille Azzurre Formation contain high amounts of liquid hydrocarbons (carbon chains C_{17} to C_{40} with a maximum at C_{29}/C_{31} and a strong odd-over-even predominance). In the low molecular weight range, a modal distribution was also observed, suggesting a mixture of thermogenic and less mature hydrocarbons. Interestingly, the fluids of the mud volcano NR7 sampled in 2009 revealed a similar distribution (Figure 29; carbon chains from C_{17} to C_{35}, with a maximum at C_{29}/C_{31}). This is in clear contrast to hydrocarbons extracted from the same mud volcanoes sampled in 2008 (cf. Heller et al. 2009) and data from Romania (Alain et al. 2006), where modal hydrocarbon distributions suggest highly mature organic matter as an origin. Note that the hydrocarbon distribution of the mud sample taken in 2009 was erroneously described to also only consist of mature hydrocarbons (Heller et al., 2011). Such distribution patterns are often present in mud volcanoes, because thermogenic cracking of these hydrocarbons leads to unimodal distributions. Thus, the relatively immature, higher molecular weight n-alkane distribution in the mud volcano NR7 (2009 campaign; Fig. 29) was surprising. However, the similarity of the shales and the mud volcano fluids clearly suggests that the underlying geological formation is the main source of the liquid hydrocarbons sampled in 2009. In contrast, in the sample taken in 2008, thermogenic hydrocarbons from the

deep reservoir prevailed, indicating strong temporal or spatial heterogeneities of liquid hydrocarbon expulsion in the Salse di Nirano. Furthermore, we found non-isoprenoidal dialkyl glycerol diethers (DAGE) in the San Polo D´Enza shale. This includes 16/16-DAGE and a suite with terminally branched pentadecane chains (i.e., *i*15/*i*15-, *i*15/*ai*15 and *ai*15/*ai*15) (see Figure 30 for structures). Additionally, we observed the isoprenoidal glycerol diether archaeol, which is common in all archaea (e.g., Blumenberg et al., 2004). Hydroxyarchaeol was not found in the shale. Several lines of evidence have shown that the anaerobic oxidation of methane takes place in the system of the Salse di Nirano mud volcanoes (Heller et al., 2011). For instance, Wrede et al. (submitted (this issue)) reported a high diversity of Archaea in mud volcano fluids, including several groups involved in the anaerobic oxidation of methane. The organisms were present in small biofilm flakes, as revealed by FISH analysis with archaea-specific and ANME 2a archaea-specific in situ hybridization probes. Support for the presence of AOM-performing microorganisms in the Salse di Nirano mud volcano came from biomarkers, which were found to be partially ^{13}C-depleted. If ^{13}C-depleted, the bacterial and archaeal biomarkers are indicative for microbial consortia performing the anaerobic oxidation of methane (AOM; Hinrichs et al., 1999; Pancost et al., 2001a, b; Thiel et al., 2001; Blumenberg et al., 2004). During AOM, ^{13}C-depleted methane carbon is transferred into the lipids of the closely operating, AOM-performing consortia of sulfate-reducing bacteria and methane-oxidizing archaea (e.g., Blumenberg et al,. 2004; Hinrichs et al., 1999; Pancost et al., 2001b). However, the origin of the majority of biomarkers in the mud volcano of the Salse di Nirano remained unexplained. Interestingly, almost all biomarkers found in the fluids of the Salse di Nirano mud volcanoes (campaign 2008 and 2009, Heller et al., 2011) were also present in the marine deposits of the Plio-Pleistocene shale (Fig. 3 and 4). The polar fraction of the mud volcano fluids revealed the same pattern of bacterial components, namely *i*15/*i*15-, *ai*15/*i*15-, *ai*15/*ai*15-, and the 16/16-DAGE (2009 campaign). Furthermore, we observed also the archaeal component archaeol (Heller et al., 2011). The fluids from the Salse di Nirano mud volcanoes, sampled in 2008, contained similar distribution patterns (Figure 30 insert; Heller et al. 2011). However, the likely SRB-derived DAGE (e.g., Pancost et al., 2001a) that were extracted from these fluids were considerably depleted in ^{13}C (e.g., *ai*15/*ai*15-

DAGE = -50.1‰) and were thus interpreted as being at least partially sourced by AOM-involved SRB (Heller et al., 2011). For the same compound from the sample taken in 2009, relatively lower concentrations and a $\delta^{13}C$ value of -35.1‰ were found (similar to the other DAGE), raising questions about the AOM-related SRB as major source in this sample. Furthermore, in the mud volcano fluids sampled in 2008 and 2009, we observed the isoprenoid glycerol diether, archaeol. The fluids from 2009 contain archaeol in much lower concentrations and trace amounts of ^{13}C-depleted sn-2-hydroxyarchaeol. Nevertheless, this suggests a high heterogeneity of the composition of extractable organic matter in Nirano and a mixture of sources for DAGE, including one independent of the anaerobic oxidation of methane. A possible explanation for some of the observed differences between the results of the biomarker studies on samples taken in 2008 and 2009 was recognized after revisiting the sampling strategies, which were apparently only incompletely described in Heller et al. (2011). In that study, both samples were reported to be taken at 1.5 m depth in the mud volcano; however, the sample from 2008 was, to a considerable extent, also obtained from the surface of the mud cone. This well explains, in comparison to the 2009 sample (Figure29; see also Heller et al. 2011), the much higher abundance of thermogenically-produced hydrocarbons seen in this study, which are, likely due to the lower density, enriched on the surface.

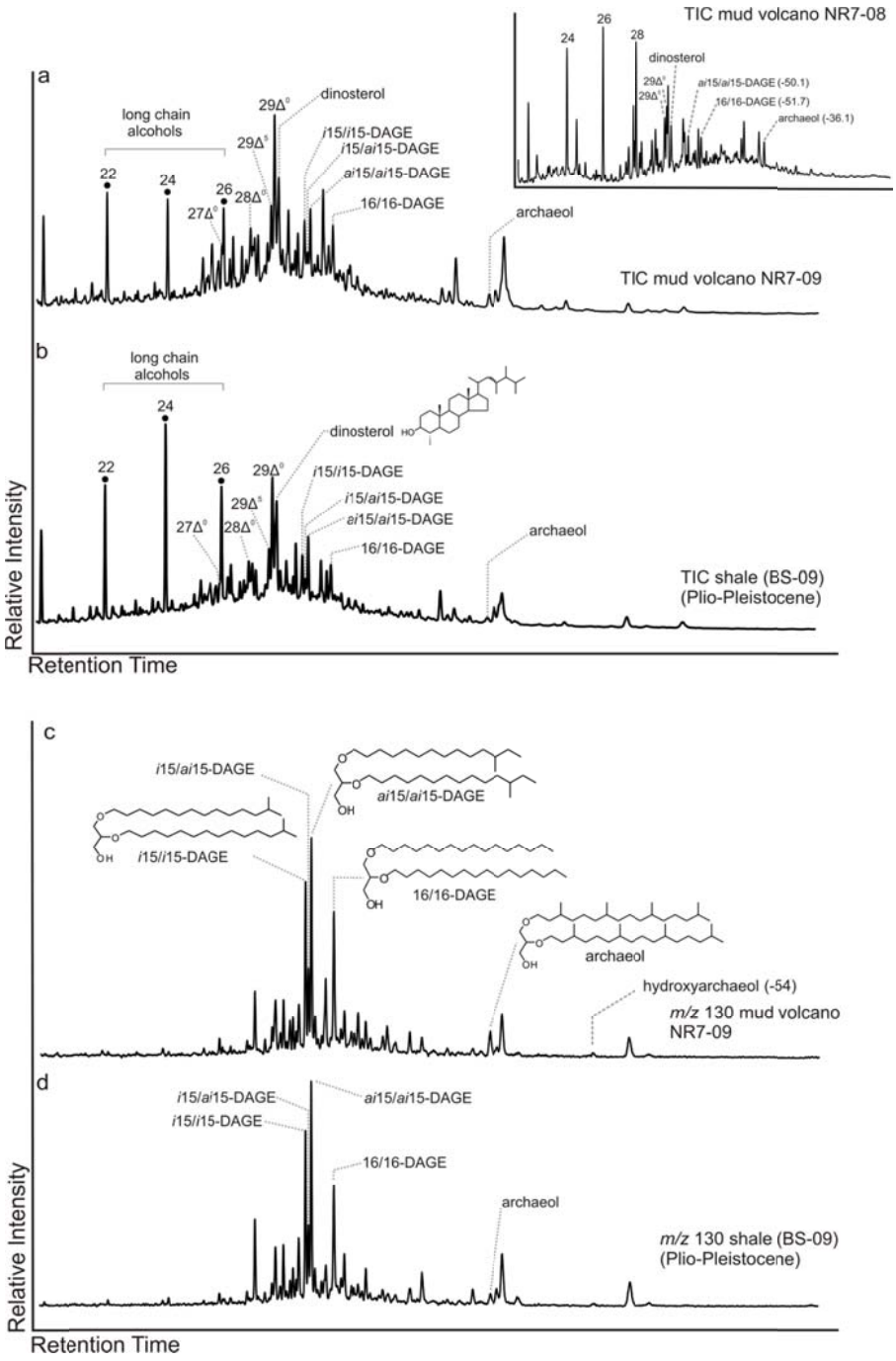

Figure 30: Lipid biomarker distribution in the mud volcano (NR7-09) and the underlying shale (BS-09). The insert shows the partial total ion chromatogram (TIC) of the mud volcano NR7 taken during the 2008 sampling campaign (Heller et al. 2011). a) and b) show partial TICs of the polar fractions of the mud volcano NR7 taken during the campaign 2009 and the Plio-Pleistocene shale of San Polo D´Enza (BS-09), respectively. c) and d) show ion chromatograms (m/z 130) from the polar fractions highlighting the distribution of dialkyl glycerol diethers (DAGE) in the mud volcano NR7 (sample campaign 2009) and the shales (BS-09) of San Polo D´Enza, respectively. 27Δ0 = cholestanol; 29Δ5 = 24-ethyl cholesterol; 29Δ0 = 24-ethyl cholestanol; dinosterol = 5α(H),4,23,24-trimethylcholest-22E-en-3β-ol.

Moreover, in this sub-setting of the mud volcano, the turbulence in the mud was relatively low, creating a more suitable environment for the slow growing, methane-oxidizing communities. This is indicated by the higher abundance of ^{13}C-depleted biosignatures of AOM-performing microorganisms observed in 2008 than in 2009, including the non-methane related DAGE source, which are abundant in both samples (Fig. 30). The comprehensive biomarker study of the underlying Plio-Pleistocene shale suggests that most of the DAGE were likely sourced from these marine deposits.

Table 12: Carbon stable isotope ratios ($\delta^{13}C$) of selected lipid biomarkers extracted from a mud volcano sample from (NR7-09) and the organic rich shales (BS-09) of San Polo D´Enza. Please note that selected $\delta^{13}C$ of biomarkers in the mud volcano sampled in 2009 have been previously published (Heller et al. 2011).

Compound	Plio-Pleistocene shale (BS-09)	Salse di Nirano mud volcano (NR7-09)
heptacosane	-	-30.5
nonacosane	-	-31.0
hentriacontane	-	-33.2
tetradecanoic acid	-33.8	-30.9
12-methyltetradecanoic acid (*ai*-15FA)	-28.1	-29.7
hexadecanoic acid	-36.3	-29.4
octadecanoic acid	-28.5	-29.2
hexacosanoic acid	-30.0	-28.9
octacosanoic acid	-31.6	-29.8
17β(H),21β(H)-bishomohopanoic acid	-29.3	-28.3
tetracosanol	-33.6	-33.8
hexacosanol	-34.6	-33.4
24-ethylcholesterol ($29\Delta^5$)	-31.6	-29.3
24-ethylcholestanol ($29\Delta^0$)	-29.5	-30.0
(5α(H),4,23,24-trimethylcholest-22E-en-3β-ol (dinosterol)	-27.5	-27.6
i15/i15-dialkyl glycerol diether (*i*15/*i*15-DAGE)	-28.0	-30.1
i15/ai15-dialkyl glycerol diether (*i*15/*ai*15-DAGE)	-34.1	-37.4
ai15/ai15-dialkyl glycerol diether (*ai*15/*ai*15-DAGE)	-40.3	-35.1
16/16-dialkyl glycerol diether (16/16-DAGE)	-37.3	-35.1
sn-2-hydroxyarchaeol	-	-54.0
17β(H),21β(H)-bishomohopanol	-31.4	-
heptatriacontadien-2-one alkenone 37:2one	-25.2	-22.9
octatriacontadien-2-one alkenone 38:2one (+Archaeol)	-29.9	-29.2

To the best of our knowledge, this is the first description of non-^{13}C-depleted to moderate ^{13}C-depleted bacterial DAGE from fossil marine shales, and their occurrence even in recent organic rich sediments is limited (Arning et al. 2009; Seidel, 2009). *i*15/*i*15-DAGE has been recently reported to be common in unknown, most likely heterotrophic and acidophilic soil bacteria (Oppermann et al. 2010), and its presence in the shale suggests a near-shore marine setting during deposition. The δ^{13}C value of -28‰ for the shale and -30‰ for the mud for *i*15/*i*15-DAGE, which is less ^{13}C-depleted than the other DAGE, supports different sources for both classes of DAGE. The knowledge of the origin of *ai*15/*ai*15-DAGE and 16/16-DAGE, in particular, is still limited. Compared to common distribution patterns of the AOM settings and our relatively moderate ^{13}C-depletion (-34.1 to -40.3‰), methane is excluded as an important carbon source; instead, heterotrophic sulfate-reducing bacteria are suggested as the primary source. An SRB-origin for these DAGE is also supported by putatively SRB-derived occurrences in recent phosphogenic, organic-rich sediments (Arning et al., 2009).Further evidence that the majority of biomarkers originate from the Plio-Pleistocene shale derived from the distribution of other biomarkers, such as 24-ethyl-cholesterol ($29\Delta^5$), 24-ethyl cholestanol ($29\Delta^0$), dinosterol, long-chain n-alcohols and n-fatty acids (not shown) and alkenones (Fig. 4). Most are of putatively aquatic origin – e.g., heptatriacontadien-2-one (37:2) and octatriacontadien-2-one (38:2) are known from haptophytic algae (Brassel et al. 1986) and the 4-methylated dinosterol from dinoflagellates (Summons et al. 1987) – together arguing for a near-shore, terrestrially-influenced marine deposit. Respective biomarkers were found to be similarly distributed in both the emitted mud at the Salse di Nirano and the shale from the underlying geological formation (Fig. 30). It appears that, during the upward migration and the residence of the fluids in the two reservoirs, they reacted with the surrounding sediments and the depositions that they passed through on their way up. This process is further promoted by the water, mud and higher hydrocarbons in the fluids, which dissolve and extract the allochthonous, immature lipid components.

6.6 Conclusions

Recent studies have shown that terrestrial mud volcanoes are settings that promote the growth of diverse hydrocarbon-degrading microorganisms (Alain et al., 2006; Heller et al. 2011; Wrede et al. submitted (this issue)). However, in addition to recent or sub-recent microbial processes, most extractable organic matter in the mud fluids has other origins; this includes mud volcanoes at our study site, the Salse di Nirano (Italy). Our comparison of lipid biomarkers of fluids seeping out of Nirano mud volcanoes with those of underlying organic-rich shale deposited during the late Pliocene – early Pleistocene demonstrated strong similarities. Various bacterial and archaeal lipid biomarkers were found in both samples. The polar fractions revealed similar patterns of bacterial biomarkers, such as *i*15/*i*15-DAGE, *ai*15/*i*15-DAGE and the *ai*15/*ai*15-DAGE, 16/16-DAGE and the archaeal component archaeol. Furthermore, we found other biomarkers, such as 24-ethyl cholesterol ($29\Delta^5$), 24-ethyl cholestanol ($29\Delta^0$), dinosterol, long chain n-alcohols and n-fatty acids, to be highly abundant and similarly distributed in both samples. Most likely, thermogenically-formed gases, liquid hydrocarbons and the mud itself act as solvents during the rise through geological formations; for this reason, mud volcanoes could act as a window into organic matter in deeply buried geological formations. Despite this fact, multiple techniques demonstrate that the fluids contain appreciable amounts of sulfate-reducing bacteria and ANME-archaea performing the anaerobic oxidation of methane. This includes previous findings of SRB-derived DAGE in the Nirano mud volcano that were considerably depleted in ^{13}C (e.g., *ai*15/*ai*15-DAGE = -50.1‰) in the sample taken in 2008 and the slightly depleted sn-2-hydroxyarchaeol in the samples taken in 2008 and 2009. One reason for the different biomarker distributions observed in samples taken in 2008 and in 2009 could be the distribution of AOM-performing organisms in terrestrial mud volcanoes. While ^{13}C-depleted biomarkers were extracted from a sample taken at a depth of only 15 cm in a relatively undisturbed milieu at the rim of the pool, those taken in 2009 were sampled at 1.5 m within the vent of the mud volcano. This suggests that the majority of AOM takes place in less turbulent upper parts of the setting. Future studies should therefore be focused on the spatial distribution of AOM-performing microorganisms in terrestrial mud volcanoes

and should be directed at the differentiation between allochthonous and autochthonous biomarker signals, perhaps with the use of intact polar lipids.

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Chapter 7

Conclusion

Two different types of methane-emitting fluid venting structures were part of this thesis: marine cold seep structures located at the NW shelf of the Black Sea and terrestrial mud volcanoes in the Northern Apennines and Sicily (Italy). The anaerobic oxidation of methane (AOM), one of the key processes of methane consumption in marine environments, takes place in both types of cold seep structures.

7.1. Black Sea

The AOM-performing microbial mats of the Black Sea cold seeps, consisting of a syntrophic association of anaerobic methane oxidizing archaea (ANME-1, -2, -3) and sulfate reducing bacteria (SRB), were used to gain deeper insight into the metabolic activities of these microorganisms. Phylogenetic and biochemical studies have postulated that the different ANME-archaea have supposedly reversed the methanogenic pathway, but until now, it was not possible to attribute the specific enzymes of the (reversed) methanogenesis to distinct organisms in the microbial mats. Therefore, immunogold-labeling techniques were used in this project to identify specific microorganisms in the microbial mats that encoded and expressed Methyl Coenzyme M reductase (MCR), one of the key enzymes of the methanogenic pathway. The application of this method has provided evidence for the expression of MCR in both the ANME-2 archaea of the black microbial mat the ANME-1 archaea of the orange-colored microbial mat. Thus, the MCR is not only encoded in the genome of the ANME-archaea, but the (reversed) methanogenic pathway is also shown to be active in both types of microorganisms.

In the Black Sea, the AOM results in the formation of distinct carbonate build-ups, which protrude into the permanent anoxic water body. LA-ICP-MS analyses of different microbial mats and the associated carbonates have shown that the different phases are enriched in Ni. The highest Ni concentrations were found in the black microbial mat and in the calcitic carbonates, whereas the lowest

concentrations are found in the orange microbial mats and aragonitic phases. Furthermore, there are extremely strong fluctuations of Ni concentrations, visible as single peaks, which often coincide with high concentrations of iron (Fe) and sulfur (S). Nickel is, among others, part of the MCR cofactor F430. Therefore, it was considered that Ni could be a geochemical tracer for the anaerobic oxidation of methane.

Microorganisms using Ni-containing enzymes for their metabolic activities are only one possible explanation for the enrichment of Ni in the active microbial mats and seep carbonates of the Black Sea. Furthermore, the formation of Ni-containing iron sulfides generated in SRB and released during cell lysis to the surrounding extracellular polymeric substances (EPS) could be another important Ni source. Additionally, Ni could be incorporated into the crystal lattice during the precipitation of carbonates. Moreover, Ni enrichment is always correlated with a depletion of ^{13}C. Hence, it was assumed that Ni correlated with negative $\delta^{13}C$-values could serve as a geochemical indicator for the AOM in recent cold seeps. In general, high Ni concentrations could occur either due to methanogenesis or AOM because both metabolic pathways are based on the same enzymes. During methanogenesis, the ^{13}C isotope will be enriched, whereas during AOM, ^{13}C gets depleted. Therefore, the Ni concentration always needs to be considered together with the stable carbon isotope data.

To confirm these observations, the fossil structure of the Montepetra cold seep (Italy) was analyzed. LA-ICP-MS performance revealed the same distribution pattern of Ni, Fe and S, as has been observed in the Black Sea methane-derived carbonates. Sites with high Ni concentrations are always correlated with a stronger ^{13}C-depletion. Previous studies of biomarker signatures have shown that AOM takes place at sites with high Ni concentrations and ^{13}C-depletion. Thus, Ni together with stable carbon isotopic ratios could act as a geochemical tracer for methanogenesis or the anaerobic oxidation of methane in both recent and fossil environments.

7.2 Terrestrial mud volcanoes

Compared with marine cold seeps, terrestrial mud volcanoes are different, although AOM also takes place in these systems. Mud volcanoes expel a three-phase mixture of gaseous and liquid hydrocarbons, water and sediment particles (mud fluid), which could serve as substrates for manifold archaeal and bacterial microorganisms. Geochemical and organo-geochemical analyses were performed on samples collected from mud volcanoes in Italy to obtain deeper insight into this complex microbial environment.

First, lipid biomarker analyses were carried out. These analyses showed that the mud fluids collected at depths from 0.15 to 1.2 m contained several slightly ^{13}C-depleted specific biomarker signatures, suggesting microbial sources that fed on the ^{13}C-depleted carbon substrates. Several dialkyl glycerol diethers (DAGE) indicative of sulfate reducing bacteria were found. Trace amounts of ^{13}C-depleted *sn*-2-hydroxyarchaeol (OH-archaeol) demonstrated that the anaerobic oxidation of methane took place in the mud volcano system. Nevertheless, that no or only slightly depleted archaeol was found shows that the vast majority of archaea present in the fluids obviously neither perform AOM nor feeds on any other ^{13}C-depleted carbon source. Other processes, such as the aerobic oxidation of methane, are more important in these systems. The lipid biomarker analyses of the organic-rich shales underlying the mud volcano area showed that in addition to recent and sub-recent processes, most extractable organic matter found in the mud fluids originates from the Plio-Pleistocene shales. The polar fraction of both the mud volcano fluids and the shales revealed similar patterns of biomarker signatures, such as *i*15/*i*15-DAGE, *ai*15/*i*15-DAGE, archaeol, 24-ethyl cholesterol and long chain fatty acids. Most likely, the fluids enriched with gaseous and liquid hydrocarbons act as a solvent during their rise through the geological formations, making mud volcanoes a window into the deep biosphere.

Most of the mud volcanoes worldwide are associated with an active petroleum system, which is also true for the mud volcanoes located in the Northern Apennines and in Sicily. As known from the Salse di Nirano, the main reservoir is located at a depth of 2 km, and a second shallower reservoir is located at a depth of 200 m. The geothermometers applied to the water samples collected for this study showed that the sources of the expelled waters (and, therefore, the gaseous

and liquid hydrocarbons of the samples analyzed in this study) were derived from a depth of 2 to 3 km, confirming the results of other studies. Furthermore, geochemical analyses of the water show that it is brackish and is mainly composed of marine connate waters, which are influenced by secondary diagenetic processes.

Geochemical and carbon isotopic studies have shown that the gas, mainly methane, was formed during the early thermogenic cracking of organic matter. Moreover, the liquid n-alkane distribution in the fluids was indicative of immature source rocks, which supports the observations made by the gas analyses. As known from other studies, secondary microbial processes such as biodegradation, secondary methanogenesis and sulfate reduction possibly linked to AOM take place in the associated petroleum reservoirs. The carbon stable isotope signatures of the carbon dioxide analyzed in this study were typically for gases that were influenced by these processes. The predominance of n-alkanes with carbon chain lengths of C_{27}, C_{29} and C_{31} deriving from immature source rocks with a high input of land plants, as well as the modal distribution patterns in the low molecular weight range, suggest a mixture of early thermogenic and less mature hydrocarbons, where the latter were most likely extracted by the rising fluids from organic-rich rocks and sediments.

As know from other areas, such as the Maccalube di Aragona, mud volcano systems have calm and more active/eruptive phases. At the Santa Barbara mud volcanoes (Caltanisetta, Sicily), for example, a paroxysmal eruption was observed in August 2008. During these active periods, the compositional and isotopic values of the expelled fluids were most likely changed. Therefore, the variations observed in the n-alkane distribution of the Salse di Nirano mud fluids collected in 2008 and 2009 could be explained by such a change between active and calm phases. The fluids expelled in 2008 showed a modal hydrocarbon distribution, suggesting more mature organic matter as their origin, whereas the fluids collected in 2009 were dominated by n-alkanes with a maximum at C_{29}/C_{31}, suggesting a mixture of early thermogenic and less mature hydrocarbons. One explanation for this result could be that during active or eruptive phases, the more mature hydrocarbons from the deep reservoir are expelled, whereas during calm phases, the fluid slowly rises to

the surface and immature hydrocarbons from the surrounding sediments are released.

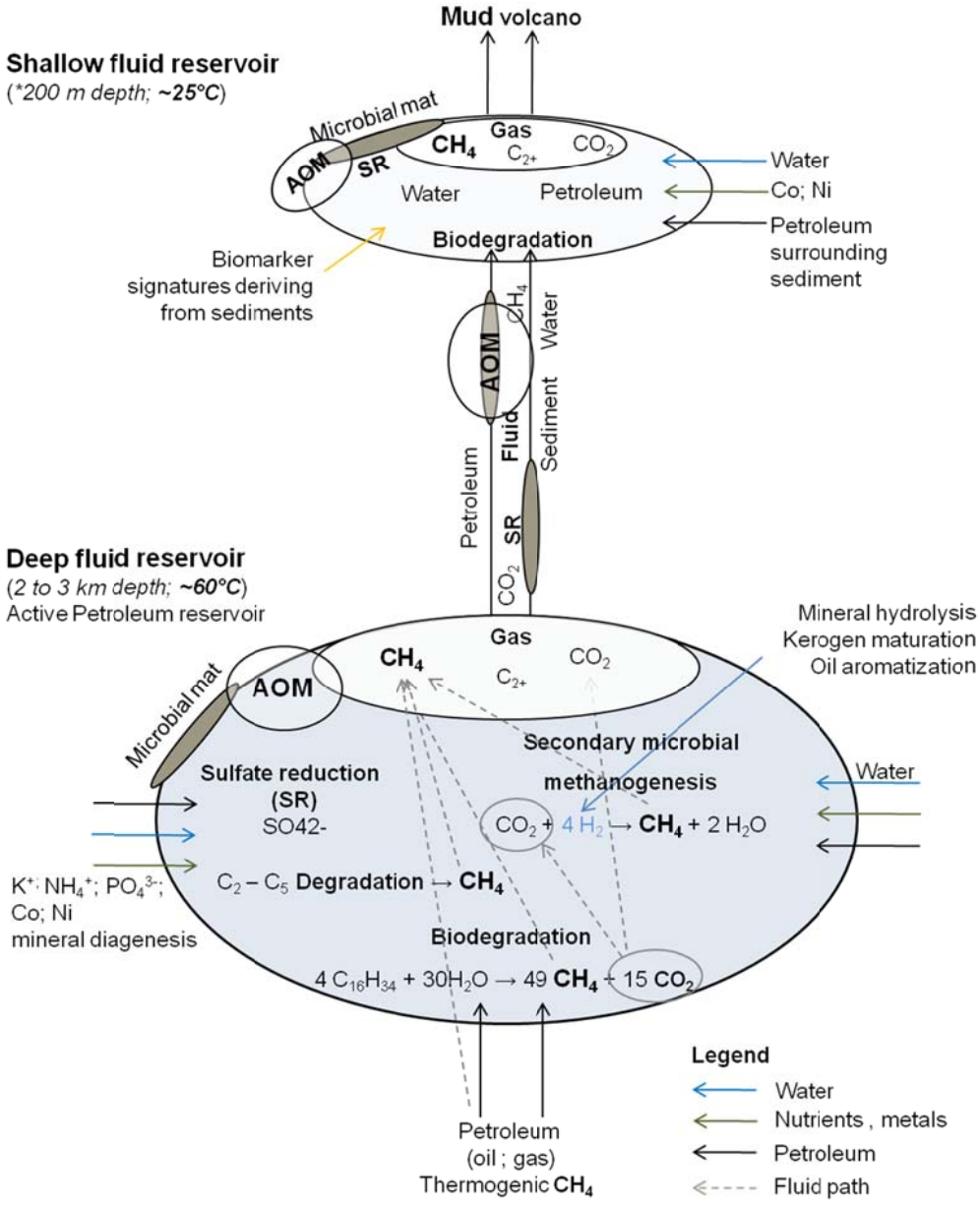

Figure 31: Schematic illustration of the putative chemistry of hydrocarbon degradation and microbial processes in active mud volcano-associated petroleum reservoirs using the example of Salse di Nirano mud volcano.

In summary, the released gaseous and liquid hydrocarbons have an early thermogenic origin influenced by secondary processes (biodegradation and secondary methanogenesis) taking place in the different reservoirs of the mud volcano system. Water has to have been available to enable microbial activity. The geothermometers show that the water temperature was approximately 60°C,

which provides ideal conditions for microbial activity. Nevertheless, the water composition itself is influenced by several secondary diagenetic processes until reaching the water-mineral equilibrium. Considering the biomarker signatures found in the expelled fluids, most of these signatures could be derived from the deep and shallow fluid reservoirs, where most of the microbial processes take place (Fig. 31). Nevertheless, some of these processes, such as the anaerobic oxidation of methane, could be executed directly in the upper surface near parts of the mud volcano system. Future studies, therefore, must focus on the spatial distribution of the different microorganisms in terrestrial mud volcanoes and must be directed toward the differentiation between allochthonous and autochthonous biomarker signatures to gain deeper insight into the terrestrial deep biosphere.