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# DIPLOMARBEIT

Titel der Diplomarbeit

„Substrate utilisation of sulfate-reducing microorganisms in a peatland“

Verfasser

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# Chapter 1. Introduction



## 1.1. Wetlands and the global climate

Climate change and anthropogenic global warming have been research topics since the 19th century, and right from the start, the greenhouse gas carbon dioxide ($CO_2$) was suspected as the major cause for raising our planet's temperature (Weart, 2008). From then on, the extent and impact of global warming have been points of debate between researchers, politicians, and laypersons. But a recent study, based on a data set of 1.6 billion temperature reports, has shown that the average global land temperature has indeed risen by nearly 1 °C in the last 60 years (Berkeley Earth team, 2011). Despite a century of research, our understanding of the global climate system is far from complete, and the future of our planet is uncertain.

It is, however, widely accepted in the scientific community that greenhouse gases (GHGs) are the main factor in global warming; the most abundant ones being water vapour, $CO_2$ and methane ($CH_4$; Wuebbles & Hayhoe, 2002). GHGs absorb infrared radiation emitted by the earth's surface, thereby trapping it in the atmosphere, which leads to heating of our planet (known as the greenhouse effect; Wuebbles & Hayhoe, 2002). $CO_2$ and $CH_4$ are both long-lived GHGs and therefore have a large and long-term impact on the climate (IPCC Fourth Assessment Report Core Writing Team, 2007). While $CO_2$ is more abundant, $CH_4$ is the more potent GHG. One mole of $CH_4$ can absorb 24 times more infrared radiation than one mole of $CO_2$ (Wuebbles & Hayhoe, 2002). Greenhouse gas emissions are categorized by their origins: natural or anthropogenic. The increased GHG emissions of the past centuries are attributed to anthropogenic sources (IPCC Fourth Assessment Report Core Writing Team, 2007), however, wetlands remain the largest source of $CH_4$.

Natural wetlands alone are estimated to be responsible for 20 % of the global $CH_4$ budget, while rice paddies are estimated to add another 12 % (Wuebbles & Hayhoe, 2002; Bridgham *et al.*, 2006). Some researchers even estimate that $CH_4$ emissions from wetlands are as high as 40 % (Liu & Whitman, 2008). Wetlands are not only a source, but also a sink and storage for organic and inorganic carbon (Limpens *et al.*, 2008). Dense plant vegetation fixes large amounts of $CO_2$ from the air. When plants die, their organic matter is degraded and mineralised by macro- and microorganisms. The balance between absorption, storage, and emission of carbon depends on the environmental conditions and the decomposer community. Currently, wetlands are primarily considered net carbon sinks, but that may change in the future (Limpens *et al.*, 2008).

Wetlands cover an approximate area of $6.0 \times 10^{12}$ m², which corresponds to 4 % of our planet's land surface (Bridgham *et al.*, 2006). The largest subtype of wetlands, the peatlands, take up an area of $3.4 \times 10^{12}$ m² (2.3 %; Bridgham *et al.*, 2006). In contrast, it is estimated that peatlands alone store a third of the global soil organic carbon (Limpens *et al.*, 2008), which makes them an important ecosystem for studying the earth's carbon fluxes. Despite this well known facts, the importance of wetlands is often neglected in climate change policies (Lenart, 2009). This only emphasises that for correct interpretation of climate data and prediction of global warming in the future, a detailed understanding of all related chemical and biological processes is required.

It has yet to be thoroughly analysed what effect global warming will have on carbon fluxes in wetlands and, in return, what role wetlands will play in future climate change. For example, do wetlands have the potential to compensate increasing $CO_2$ emissions from anthropogenic sources by acting as carbon sinks? Will temperature increases change or destroy this complex environments? To be able to answer such questions, it is vital to understand biogeochemical cycles in wetlands, the roles and interactions of the key organisms, and how they will be affected by external and internal factors. This study focuses on sulfate-reducing microorganisms (SRMs), which play an indirect, but important role in reducing $CH_4$ emission from peatlands.

## 1.2. Microbial processes in peatlands

High groundwater levels in peatlands create an oxic-anoxic interface zone, followed by a large anoxic environment in greater depths. In the absence of oxygen the degradation pattern of organic matter depends on the availability of alternative electron acceptors. Typical electron acceptors found in peatlands are sulfate ($SO_4^{2-}$), nitrate, and iron(III) (e.g. Blodau *et al.*, 2007; Knorr *et al.*, 2009). In the absence of these electron acceptors, organic compounds are broken down by fermentation followed by methanogenesis which leads to the formation of $CO_2$ and $CH_4$ (Figure 1.1, Table 1.1). $CH_4$ diffuses towards the surface and is either reoxidised by methanotrophs in the oxidised soil layer or is released into the atmosphere and therefore removed from the peatland's carbon cycle (Le Mer & Roger, 2001; Smemo & Yavitt, 2011).

$CO_2$ is produced by all domains of life, but the process of methanogenesis is exclusive to the *Archaea*, specifically to five orders within the *Euryarchaeota* (Thauer *et al.*, 2008; Ferry, 2010). These methanogens are generally separated in two groups, those with cytochromes and those without, and depending on the presence of cytochromes they can grow on different substrates (Thauer *et al.*, 2008). The major substrates for methanogens are molecular hydrogen ($H_2$) and $CO_2$, formate, and acetate (see Table 1.1), but they can also grow on other molecules with methyl groups, i.e. methanol, methylamines or dimethylsulfide (Thauer, 1998; Ferry, 2010).

**Table 1.1** Sulfate-reducing, methanogenic and anaerobic oxidation of $CH_4$ reactions (Rabus *et al.*, 2006; Muyzer & Stams, 2008; Smemo & Yavitt, 2011). Methanogens can utilise formate by converting it to $H_2/CO_2$ (Thauer *et al.*, 2008).

| Equation | $\Delta G^{0\prime}$ or $\Delta G'$ (kJ reaction$^{-1}$) |
|---|---|
| *Methanogenic reactions* | $\Delta G^{0\prime}$ |
| $4\ H_2 + HCO_3^- + H^+ \longrightarrow 3\ H_2O + CH_4$ | −135.6 |
| $Acetate^- + H_2O \longrightarrow HCO_3^- + CH_4$ | −31.0 |
| *Sulfate-reducing reactions* | $\Delta G^{0\prime}$ |
| $4\ H_2 + SO_4^{2-} + H^+ \longrightarrow 4\ H_2O + HS^-$ | −151.9 |
| $4\ Formate^- + SO_4^{2-} + H^+ \longrightarrow 4\ HCO_3^- + HS^-$ | −146.6 |
| $Acetate^- + SO_4^{2-} \longrightarrow 2\ HCO_3^- + HS^-$ | −47.6 |
| $Lactate^- + 0.5\ SO_4^{2-} \longrightarrow Acetate^- + HCO_3^- + 0.5\ HS^- + 0.5\ H^+$ | −80.2 |
| $Propionate^- + 0.75\ SO_4^{2-} \longrightarrow Acetate^- + HCO_3^- + 0.75\ HS^- + 0.25\ H^+$ | −37.7 |
| $Butyrate^- + 0.5\ SO_4^{2-} \longrightarrow 2\ Acetate^- + 0.5\ HS^- + 0.5\ H^+$ | −27.8 |
| *Reaction for anaerobic oxidation of $CH_4$* | $\Delta G'$[a] |
| $CH_4 + SO_4^{2-} \longrightarrow HCO_3^- + HS^- + H_2O$ | −19.3 to −14.6 |
| $5\ CH_4 + 8\ NO_3^- + 8\ H^+ \longrightarrow 5\ CO_2 + 4\ N_2 + 14\ H_2O$ | −372.8 to −337.1 |
| $CH_4 + Fe(OH)_3 \longrightarrow HCO_3^- + FeCO_3 + 3\ H_2O$ | −123.0 to −115.0 |

[a]Calculated for standard conditions and ion concentrations found in different peatlands (Smemo & Yavitt, 2011).

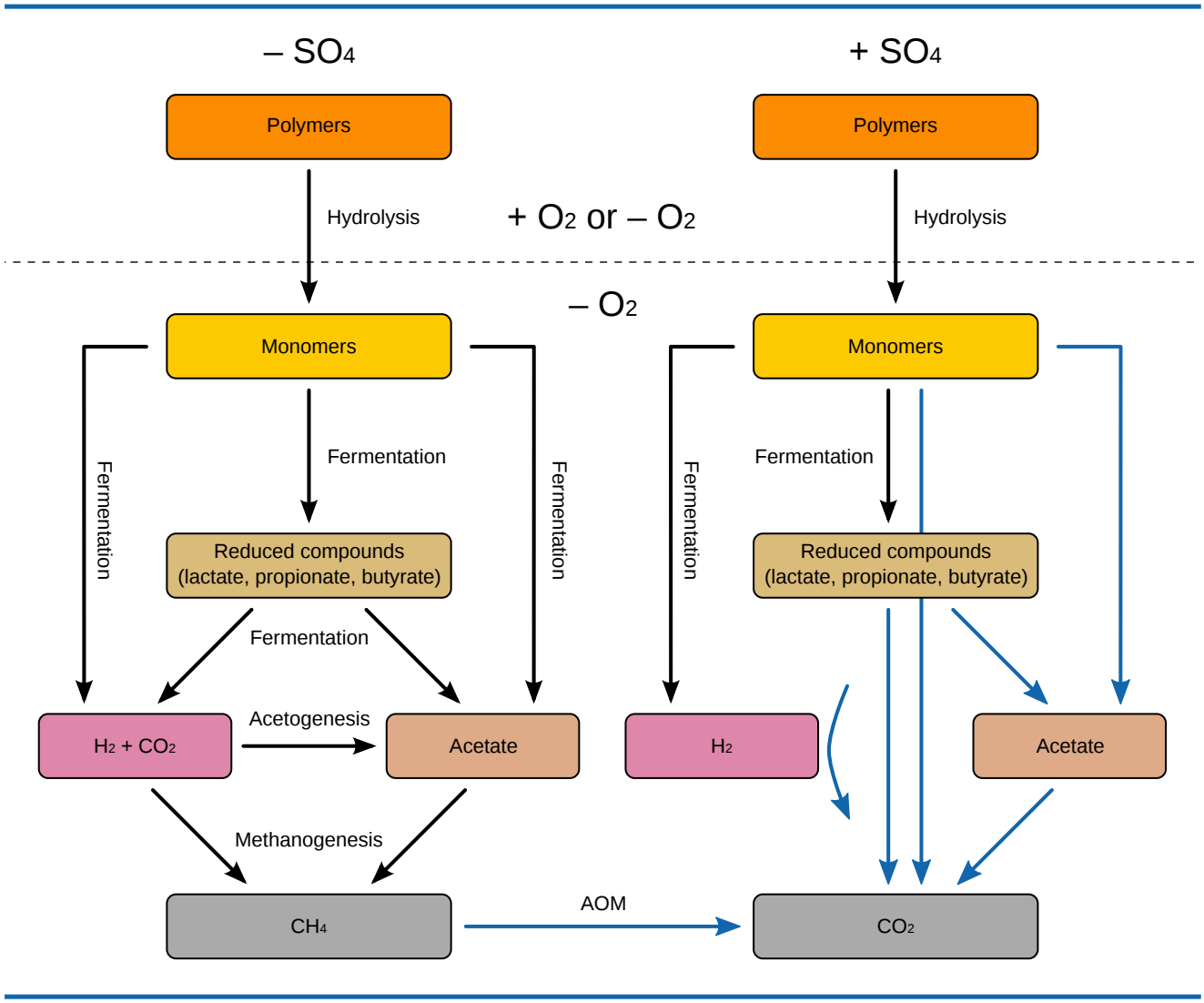


**Figure 1.1** Schematic representation of organic degradation patterns by microbial communities in anoxic environments in the absence and presence of sulfate (based on Figure 3 from Muyzer & Stams, 2008). *AOM* anaerobic oxidation of $CH_4$ (Smemo & Yavitt, 2011; Thauer, 2011). Sulfate-reducing reactions are shown as blue arrows ($SO_4^{2-} \longrightarrow HS^-$).

SRMs, on the other hand, can utilise a much broader range of electron donors than methanogens. Common electron donors in dissimilatory sulfate reduction are fatty acids, lactate and $H_2$ (see Table 1.1), but also ethanol, succinate, fumarate, malate, fructose, glucose and phenyl-substituted organic acids (Rabus *et al.*, 2006). Based on the end product of these different physiological pathways, it is possible to divide SRMs into two groups:

*Complete oxidisers*
: The electron donor is completely mineralised to $CO_2$ or $H_2O$. These SRMs can be further partitioned by which metabolic pathway they use for mineralisation (Rabus *et al.*, 2006).

*Incomplete oxidisers*
: Substrates are broken down to acetate, which is then excreted. This can be advantageous, because at substrate surplus, incomplete oxidation yields more energy than complete oxidation. Common sulfate-reducing reactions are shown in Table 1.1.

Most SRMs can also utilise thiosulfate, sulfite or elemental sulfur as terminal electron acceptors. In the absence of inorganic sulfur species, some SRMs are also capable of alternative metabolic pathways, for example fermentation of fumarate or malate, or reduction of nitrate, iron(III), arsenate(V),

chromate(VI) or uranium(VI) (Rabus *et al.*, 2006). The numbers of fatty acid molecules which are consumed in a reaction with one molecule of sulfate are shown in Table 1.2. In case of incomplete oxidation, more substrates can be turned over per sulfate molecule.

**Table 1.2** Number of short-chain fatty acid molecules which are turned over with one molecule of sulfate. Values are for sulfate reduction with complete oxidation to $CO_2$ and incomplete oxidation to acetate.

| Substrate | Complete oxidation | Incomplete oxidation |
|---|---|---|
| Formate | 4.00 | — |
| Acetate | 1.00 | — |
| Lactate | 0.67 | 2.00 |
| Propionate | 0.57 | 1.33 |
| Butyrate | 0.40 | 2.00 |

SRMs are a polyphyletic group with members both in the domains *Bacteria* and *Archaea* (Rabus *et al.*, 2006). They are members of the bacterial phyla/classes *Deltaproteobacteria*, *Firmicutes*, *Nitrospirae*, and *Thermodesulfobacteria*; and in the archaeal phyla *Crenarchaeota* and *Euryarchaeota*. Most recognised SRMs belong either to the *Deltaproteobacteria* or the *Firmicutes* (Wagner *et al.*, 2005; Muyzer & Stams, 2008). SRMs associated with methanotrophic archaea (see anaerobic oxidation of $CH_4$) are exclusively members of the *Deltaproteobacteria* (Thauer, 2011). Their grouping is solely based on their ability for dissimilatory sulfate reduction. Since the 16S rRNA is no suitable phylogenetic marker for detection of polyphyletic taxa, a functional marker was established, the *dsrAB* genes (encoding α and β subunits of the dissimilatory (bi)sulfite reductase; Wagner *et al.*, 2005). Phylogenetic information gained by using the *dsrAB* marker genes has to be interpreted with care, since these genes have undergone lateral gene transfer (Wagner *et al.*, 2005). Also, the presence of *drsAB* genes does not prove that an organisms has the ability to reduce sulfate. Obtained *dsrAB* sequences could be from untranscribed pseudogenes (Wagner *et al.*, 2005), but even transcription does not prove sulfate-reducing activity (Imachi *et al.*, 2006). SRMs are present in most aquatic habitats and grow at a broad temperature range (Rabus *et al.*, 2006). If sulfate is present in an anoxic environment, methanogens and SRMs can interact in several different ways (Muyzer & Stams, 2008):

*Direct competition over common substrates*
: Methanogens, as well as some SRMs, can utilise $H_2$, formate, or acetate, which results in direct nutrient competition by those two groups. Table 1.1 shows the $\Delta G^{0\prime}$ values from utilising these substrates by either methanogenesis or sulfate reduction. SRMs have the advantage from an energy standpoint with both substrates and can outcompete methanogenic archaea. But this can be a very slow process, since the difference in $\Delta G^{0\prime}$ values is not very high.

*Inhibition of acetate production*
: Only a limited number of substrates can be used for methanogenesis, one of them being acetate. SRMs, on the other hand, can degrade a lot of different substrates. Complete oxidation of short-chain fatty acids and other monomers directly to $CO_2$ circumvents the production of acetate and therefore removes an important substrate for methanogenic archaea.

*Syntrophy*
: Incomplete oxidation to acetate is common among SRMs. The produced acetate can be further broken down by complete-oxidizing SRMs (see *Direct competition over common substrates*) or fuel methanogenesis. The combination of incomplete oxidisers and acetotrophic methanogens can lead to a syntrophic relationship. Another type of syntrophy was reported previously, where SRMs (e.g. *Syntrophobacter*) releases reducing equivalents to methanogens (Wallrabenstein *et al.*, 1994; Wallrabenstein *et al.*, 1995; Harmsen *et al.*, 1998).

*Anaerobic oxidation of $CH_4$*

In anoxic environments, $CH_4$ can be oxidised by dissimilatory sulfate reduction (or other dissimilatory pathways; Thauer, 2011). Coupling of sulfate reduction with methanotrophy is well studied in marine sediments and is performed by syntrophic communities consisting of methanotrophic archaea and SRMs (Valentine, 2002; Thauer, 2011). Smemo & Yavitt (2011) suggest that anaerobic oxidation of $CH_4$ could also be going on in peatlands.

Sulfate exits at low concentrations in peatlands (e.g. Küsel & Alewell, 2004; Reiche *et al.*, 2009) and can be rapidly turned over (Blodau *et al.*, 2007; Knorr & Blodau, 2009). This means that continuous sulfate reduction, and as a result suppression of methanogenesis, is only possible if the sulfate pools are regularly replenished. New sulfate is introduced into peatlands through acid precipitation, groundwater, or organic deposition (Muyzer & Stams, 2008). Pollution from fossil fuel combustion adds massive amounts of sulfur dioxide ($SO_2$) to the atmosphere, which is then converted to sulfuric acid ($H_2SO_4$) by chemical reactions in the air and introduced into peatlands by precipitation (Muyzer & Stams, 2008). It has been projected that through population growth and energy consumption in Asia in the next decades, $SO_2$ pollution will increase and continue to be an important factor in peatland and global carbon/sulfur fluxes (Streets & Waldhoff, 2000; Gauci *et al.*, 2004).

However, introduction of external sulfate can not account for sulfate turnover rates measured in previous studies, which points towards an internal recycling of sulfate. Internal sulfur cycling was reported at different scales and under oxic and anoxic conditions, either by chemically or microbial processes. While the mechanisms are not completely understood or their existence in peatlands proven, evidence suggests that a part of the sulfide pool is recycled to sulfate either via elemental sulfur or thiosulfate (Blodau *et al.*, 2007; Pester *et al.*, 2012).

## 1.3. Model habitat Schlöppnerbrunnen

To understand the ecological and geological significance of peatlands in the global climate, the study of selected model ecosystems is imperative. The Schlöppnerbrunnen fen system is a long-term experimental field site, where extensive research was done in different scientific fields over the past decades, for example biogeochemical studies (Knorr *et al.*, 2009; Knorr & Blodau, 2009) or targeted molecular biology approaches (Loy *et al.*, 2004; Pester *et al.*, 2010).

This model system is located in the Lehstenbach catchment in the Fichtelgebirge mountains (Northeastern Bavaria, Germany) and consists of two minerotrophic fens. Schlöppnerbrunnen I (50°08′14″N, 11°53′07″E) is located in the upper part of the catchment and is water saturated (but can become dry after prolonged hot weather), whereas Schlöppnerbrunnen II (50°08′38″N, 11°51′41″E) is located in the lower part of the catchment and is permanently water saturated (Loy *et al.*, 2004) with average yearly groundwater table depths of approximately 0.2 and 0.1 m, respectively (Küsel *et al.*, 2008). This creates a small oxygenated zone, followed by a larger zone, where oxic and anoxic conditions switch, depending on the current water level. Soil solution pH values vary between between 4 and 6 (Loy *et al.*, 2004; Schmalenberger *et al.*, 2007) and the annual average air temperature is about 5 °C (Loy *et al.*, 2004; Küsel *et al.*, 2008; Palmer *et al.*, 2010).

Sulfate concentrations up to 200–300 µmol $L^{-1}$ were measured in the previous studies (Loy *et al.*, 2004, Schmalenberger *et al.*, 2007). In previous studies, standing pools of formate, acetate, and lactate were around 100 µmol $L^{-1}$, while propionate and butyrate concentrations were typically much lower or not detectable (Schmalenberger *et al.*, 2007; Küsel *et al.*, 2008). Despite small standing pools of sulfate, considerable sulfate reduction rates have been detected in previous studies (up to 340 nmol (g soil w. wt.)$^{-1}$ $day^{-1}$; Knorr & Blodau, 2009; Knorr *et al.*, 2009). Periodic supply of sulfate

to the Schlöppnerbrunnen fens is guaranteed through deposited sulfate on higher ground, which is washed down during rain. Those depositions are the result of unfiltered coal burning by power stations in eastern Europe in the last century (Moldan & Schnoor, 1992; Berge *et al.*, 1999). Suppression of methanogenesis caused by substrate or sulfate supplementation in Schlöppnerbrunnen soil has been shown. A nearly 80 % reduction of $CH_4$ emissions was observed in anoxic incubations with sulfate (Loy *et al.*, 2004), while incubations amended with acetate, propionate, or butyrate inhibited methanogenesis by 80 % to 96 % (Horn *et al.*, 2003).

In the last decade, a total of 53 novel and uncultured species-level *dsrAB* operational taxonomic units (OTUs) were found in Schlöppnerbrunnen (Loy *et al.*, 2004; Schmalenberger *et al.*, 2007; Pester *et al.*, 2010; Steger *et al.*, 2011), making it an interesting habitat for the research on sulfate reduction in peatlands. A recent study by Pester *et al.* (2010) aimed to identify microorganisms responsible for sulfate reduction in Schlöppnerbrunnen II. Anoxic incubations at 14 °C supplemented with sulfate and $^{13}$C-labelled substrates (at *in situ* concentrations) were done, followed by stable isotope probing (SIP). A combination of terminal restriction fragment length polymorphism and clone libraries could identify the genus *Desulfosporosinus* as the most active SRM group under the conditions supplied. *Desulfosporosinus*-specific primer pairs were designed and quantitative PCR (qPCR) was used to determine their natural abundance, and monitor the increase of 16S rRNA gene copy numbers in the six-month long incubation experiment. Interestingly, the natural abundance of *Desulfosporosinus* species was only 0.006 % and therefore they are part of the "rare biosphere". The microbial rare biosphere describes low-abundant (but not necessarily inactive or unimportant) species, which would be assigned to the tail of a rank abundance curve (Pedrós-Alió, 2006; Sogin *et al.*, 2006; Pedrós-Alió, 2007). Pester *et al.* (2010) additionally estimated cell-specific sulfate reduction rates for this *Desulfosporosinus* population, which were in the same range as reported for pure cultures, making them members of the Schlöppnerbrunnen *active* rare biosphere.

## 1.4. The genus *Desulfosporosinus*

The genus *Desulfosporosinus* is assigned to the taxonomic order *Clostridiales*, within the phylum of the *Firmicutes*. Members of this genus are strictly anaerobic, have the ability to form endospores and a DNA G + C content between 37 and 47 mol%. Microscopy of *Desulfosporosinus* strains revealed curved, rod-shaped bacteria with a cell diameter of 0.4–1.2 µm and a length of 2.5–5.5 µm, which can also have flagella. With the exception of *D. auripigmenti*, they stain gram-negative (Spring & Rosenzweig, 2006; Vatsurina *et al.*, 2008; Lee *et al.*, 2009; Alazard *et al.*, 2010). Currently, seven cultivated species (with representatives in culture collections) belong to the genus *Desulfosporosinus* (Table 1.3).

**Table 1.3** Cultivated *Desulfosporosinus* species with representatives in culture collections.

| Name | Type strain | Isolated from | Reference |
|---|---|---|---|
| *D. acidiphilus* | DSM 22704 | Acid mine drainage sediment | Alazard *et al.*, 2010 |
| *D. auripigmenti* | DSM 13351 | Freshwater sediment | Newman *et al.*, 1997, Stackebrandt *et al.*, 2003 |
| *D. hippei* | DSM 8344 | Permafrost soil | Vatsurina *et al.*, 2008 |
| *D. lacus* | DSM 15449 | Freshwater sediment | Ramamoorthy *et al.*, 2006 |
| *D. meridiei* | DSM 13257 | Gasolene-contaminated groundwater | Robertson *et al.*, 2001 |
| *D. orientis* | DSM 765 | Soil | Campbell & Postgate, 1965, Stackebrandt *et al.*, 1997 |
| *D. youngiae* | DSM 17734 | Constructed wetland / acid mine drainage sediment | Lee *et al.*, 2009 |

*Desulfosporosinus* species can autotrophically grow with $H_2$ or gain energy by oxidation of formate or incomplete oxidation of lactate, butyrate, and pyruvate to acetate. They can also use $H_2$ plus acetate or yeast extract for electron donors, but they could not utilise acetate alone or propionate under the conditions tested (Spring & Rosenzweig, 2006). All members of this genus can use sulfate and thiosulfate as electron acceptors and some species can additionally use elemental sulfur, sulfite, fumarate, nitrate, iron(III) or arsenate(V) (Newman *et al.*, 1997; Liu *et al.*, 2004; Ramamoorthy *et al.*, 2006; Spring & Rosenzweig, 2006). Alternatively, it has been shown that most *Desulfosporosinus* species have the ability for fermentative growth with lactate or pyruvate (Spring & Rosenzweig, 2006; Vatsurina *et al.*, 2008).

## 1.5. Aims of this study

The aims of this study can be summarised as follows:

i. Setup, maintain, and regularly sample anoxic microcosm incubations of Schlöppnerbrunnen soil slurries, supplemented with sulfate and common electron donors used by SRM, over the course of eight weeks ("long-term") to determine the ecophysiology of SRMs and the active rare biosphere member *Desulfosporosinus*. This is the basis for all following aims.

ii. Measure sulfate and substrate concentrations from the long-term incubation experiment and additionally from a similar one-week incubation experiment ("short-term"), to reveal sulfate and single-substrate utilisation profiles of the Schlöppnerbrunnen microbial community in a fine and coarse temporal range. Determine sulfate reduction rates by using sulfate concentrations from the short-term incubation experiment and data from a cooperation partner at the University of Bayreuth (Germany).

iii. Develop, evaluate, and optimise a protocol for nucleic acids extraction from peatland soil suitable to determine 16S rRNA gene and transcript copy numbers with (reverse transcription) quantitative PCR targeting either "total" *Bacteria* & *Archaea* or *Desulfosporosinus*.

iv. Apply the nucleic acids extraction pipeline to selected slurry samples for proof of principle and to gain first insights into metabolic activity and growth of the *Desulfosporosinus* population in incubations with different substrates.

v. Enrich Schlöppnerbrunnen SRMs from soil slurry samples with classical cultivation techniques, with the final goal to isolate a novel SRM species or a *Desulfosporosinus* species indigenous to the Schlöppnerbrunnen fens.

The incubations in the study of Pester *et al.* (2010) were done with a mix of different electron donors (formate, acetate, lactate, and propionate), which were all turned over. Therefore it was not possible to determine, which of these were being used by the *Desulfosporosinus* species or other SRM. This study aims to answer this question with incubations of single-substrate microcosm soil slurries. Besides the substrates used in the previous study, a set of incubations with butyrate were done as well. No preincubation without substrate amendment (like in the previous SIP-study) was done, since every additional incubation step affects the microbial community and therefore their responses may differ compared to their natural environment.

The qPCR assays for 16S rRNA gene quantification were already established by Pester *et al.* (2010). This study extended the protocol to include the quantification of reverse transcribed RNAs. Therefore extensive evaluation and optimisation of the nucleic acids extraction and gene/transcript quantification methods was necessary. Based on the substrate utilisation profiles, the metabolic activity and growth of *Desulfosporosinus* sp. in one selected microcosm was monitored by quantifying 16S rRNA transcript copies and comparing it to the 16S rRNA gene copy numbers. Because soil is a very heterogeneous

environment, the number of 16S rRNA genes of *Desulfosporosinus* species was also normalised against total counts of 16S rRNA genes from all *Bacteria* & *Archaea*.

# Chapter 2. Materials and Methods



All laboratory work was done at the Department of Microbial Ecology (University of Vienna) with the following exceptions:

- Bead beating was sometimes done at the Department of Genetics in Ecology (University of Vienna).
- The Tecan Infinite M200 microplate reader used for DNA and RNA quantification is located at the Department of Terrestrial Ecosystem Research (University of Vienna).

A comprehensive list of laboratory suppliers is given in the appendix, with abbreviated company names, which are used within this document from here on (Table A.1).

## 2.1. Equipment and software

**Table 2.1** Technical equipment used, including corresponding software.

| Machine (type) | Software (version) | Manufacturer |
|---|---|---|
| P/ACE MDQ Molecular Characterization System (capillary electrophoresis instrument) | 32 Karat (7.0) | Beckman Coulter |
| iCycler™ (thermal cycler) | — | Bio-Rad |
| iCycler™ iQ Real-Time PCR Detection System (thermal cycler) | iCycler™ iQ (3.1) | Bio-Rad |
| Sub-Cell GT (agarose gel electrophoresis system) | — | Bio-Rad |
| Sub-Cell GT UV-Transparent Gel Tray | — | Bio-Rad |
| PowerPac Basic (electrophoresis power supply) | — | Bio-Rad |
| UST-C30M-8R (UV transilluminator) | Argus X1 (4.1) | Biostep |
| M107 High Specification (visible spectrophotometer) | — | Camspec |
| 5804 R (microcentrifuge) | — | Eppendorf |
| Mikro 20 (microcentrifuge) | — | Hettich Lab Technology |
| Mikro 22 R (microcentrifuge) | — | Hettich Lab Technology |
| Rotina 35 R (microcentrifuge) | — | Hettich Lab Technology |
| Wildfire smartphone (digital camera) | Android (2.2) | HTC |
| Hybridisation oven | — | Memmert |
| Milli-Q Water Purification System | — | Millipore |
| Analytical Plus (analytical balance) | — | Ohaus |
| C-5050 Zoom (digital camera, used with UV transilluminator) | — | Olympus |
| UV Sterilising PCR Workstation | — | PEQLAB |
| BIO 101/Savant FastPrep™ FP120 (cell lysis/homogenizer) | — | Qbiogene |
| MIR-153 (cooled incubator) | — | Sanyo |
| BL3100 (balance) | — | Sartorius |
| BL6100 (balance) | — | Sartorius |
| Infinite® M200 (microplate reader) | i-control™ (1.6) | Tecan |
| NanoDrop ND-1000 (UV-visible spectrophotometer) | ND-1000 (3.2) | Thermo Fisher Scientific |
| inoLab® pH Level 1 (pH meter) | — | WTW |
| LSM 510 META (confocal laser scanning microscope) | LSM 510 (3.2) | Zeiss |
| Axioplan 2 imaging (epifluorescence microscope) | AxioVision (4.7) | Zeiss |
| AxioCam HRc (digital camera, used with epifluorescence microscope) | — | Zeiss |

Statistical analysis was done with the R programming language (versions 2.13 and 2.14; R Development Core Team, 2011) or with LibreOffice Calc (version 3.4; LibreOffice contributors and/or their affiliates, 2011). Figures in this document were prepared with the R programming language, the R package ggplot2 (version 0.8.9; Wickham, 2009), Inkscape (version 0.48; Inkscape contributors, 2011) and/or the GNU Image Manipulation Program (version 2.6; Kimball *et al.*, 2010).

If necessary, the following steps were done with the GNU Image Manipulation Program to prepare images of agarose gels: conversion to the greyscale colour mode, colour inversion, adjusting the contrast, image cropping and rotation. 16S rRNA sequence analysis was done with ARB (version 5.1; Ludwig *et al.*, 2004).

## 2.2. Consumables and molecular biology kits

RNase-, DNase- and (human) DNA-free tubes and plates from new, sealed packages were used. PCR consumables were additionally radiated with UV light, immediately before pipetting.

**Table 2.2** General consumables and plasticware.

| Consumable | Manufacturer |
|---|---|
| Optical sealing tape (for use with iCycler™ iQ) | Bio-Rad |
| 0.2 mL PCR tubes | Biozym |
| 0.6 mL reaction tubes | Biozym |
| Pipette tips with filter, various sizes | Biozym |
| Inject™-F syringes, 1 mL | Braun |
| Omnifix®-F syringes, various sizes (1–60 mL) | Braun |
| Sterican® needles, various sizes | Braun |
| 50 mL reaction tubes | Carl Roth |
| Pipette tips, various sizes | Carl Roth |
| PCR plates, 96-well, skirted, blue or red (for use in standard PCR) | Eppendorf |
| PCR film (adhesive) | Eppendorf |
| 1.5 mL reaction tubes | Greiner Bio-One |
| 2 mL reaction tubes | Greiner Bio-One |
| 15 mL reaction tubes | Greiner Bio-One |
| 50 mL reaction tubes | Greiner Bio-One |
| Microplates, 96-well, flat bottom, chimney, black | Greiner Bio-One |
| FastPrep™ Lysing Matrix A tubes (used without ceramic sphere) | MP Biomedicals |
| FastPrep™ Lysing Matrix E tubes | MP Biomedicals |
| PCR plates, 96-well, semi-skirted, colourless (for use in qPCR) | PEQLAB |
| Sterile syringe filters | VWR |
| Sterile gas filters | Whatman |

**Table 2.3** Molecular biology kits

| Kit | Manufacturer |
|---|---|
| TURBO DNA-free™ Kit | Applied Biosystems |
| Quant-iT™ PicoGreen® dsDNA Reagent and Kit | Invitrogen |
| Quant-iT™ RiboGreen® RNA Reagent and Kit | Invitrogen |
| SuperScript® VILO™ cDNA Synthesis Kit | Invitrogen |
| SuperScript™ III First-Strand Synthesis System for RT-PCR | Invitrogen |
| Platinum® SYBR® Green qPCR SuperMix-UDG | Invitrogen |
| RNase ONE Ribonuclease | Promega |
| AllPrep DNA/RNA Mini Kit | QIAGEN |
| RNase-Free DNase Set (for AllPrep DNA/RNA Mini Kit) | QIAGEN |
| QIAquick PCR Purification Kit | QIAGEN |
| OneStep™ PCR Inhibitor Removal Kit | Zymo Research |

## 2.3. Primers and probes

Primers listed in Table 2.4 were used for PCR and qPCR (see Section 2.10). M13 primer sequences are included in the multiple cloning site of the vector used in the TOPO® TA Cloning kits (Invitrogen). The general *Bacteria* & *Archaea* and *Desulfosporosinus* 16S rRNA gene primers were used for 16S rRNA gene amplification. In early qPCR assays, a modified version of the primer 1389F was used (1389Farch), in the final qPCR analysis a combination of both (1389Fmix). It is noted if the 1389Farch primer was used, otherwise the 1389Fmix primer was used.

**Table 2.4** Primers used in standard and quantitative real-time PCR.

| Name | Sequence | Length (nt) | Specificity | Reference |
|---|---|---|---|---|
| M13 Forward | 5′-GTA AAA CGA CGG CCA G-3′ | 16 | MCS of many vectors | TOPO® TA Cloning manual |
| M13 Reverse | 5′-CAG GAA ACA GCT ATG AC-3′ | 17 | MCS of many vectors | TOPO® TA Cloning manual |
| 1389F | 5′-TGT ACA CAC CGC CCG T-3′ | 16 | Most *Bacteria* (16S rRNA genes) | Modified from Loy *et al.*, 2002 |
| 1389Farch | 5′-TGC ACA CAC CGC CCG T-3′ | 16 | Most *Archaea* (16S rRNA genes) | Modified from Loy *et al.*, 2002 |
| 1389Fmix | 5′-TGY ACA CAC CGC CCG T-3′ | 16 | Most *Bacteria* and *Archaea* (16S rRNA genes) | (Combination of 1389F and 1389Farch) |
| 1492R | 5′-GGY TAC CTT GTT ACG ACT T-3′ | 19 | Most *Bacteria* and *Archaea* (16S rRNA genes) | Loy *et al.*, 2002 |
| DSP603F | 5′-TGT GAA AGA TCA GGG CTC A-3′ | 19 | *Desulfosporosinus* (16S rRNA genes) | Pester *et al.*, 2010 |
| DSP821R | 5′-CCT CTA CAC CTA GCA CTC-3′ | 18 | *Desulfosporosinus* (16S rRNA genes) | Pester *et al.*, 2010 |

Probes listed in Table 2.5 were used for fluorescence *in situ* hybridization (see Section 2.12). Probe combinations EUB338mix, Delta495mix and LGC354mix were equimolar mixtures of all their different versions. The online tool probeBase[1] (Loy *et al.*, 2007) was used to select appropriate probes. All primers and probes where synthesised by Thermo Fischer Scientific.

**Table 2.5** Probes used for rRNA-targeted fluorescence *in situ* hybridization. All probes were hybridised at a formamide concentrations of 35 %.

| Name | Sequence | Length (nt) | Specificity | Reference |
|---|---|---|---|---|
| EUB338 | 5′-GCT GCC TCC CGT AGG AGT-3′ | 18 | Most *Bacteria* | Amann *et al.*, 1990 |
| EUB338 II | 5′-GCA GCC ACC CGT AGG TGT-3′ | 18 | *Planctomycetales* | Daims *et al.*, 1999 |
| EUB338 III | 5′-GCT GCC ACC CGT AGG TGT-3′ | 18 | *Verrucomicrobiales* | Daims *et al.*, 1999 |
| NONEUB | 5′-ACT CCT ACG GGA GGC AGC-3′ | 18 | Negative control | Wallner *et al.*, 1993 |
| LGC354A | 5′-TGG AAG ATT CCC TAC TGC-3′ | 18 | *Firmicutes*[a] | Meier *et al.*, 1999 |
| LGC354B | 5′-CGG AAG ATT CCC TAC TGC-3′ | 18 | *Firmicutes*[a] | Meier *et al.*, 1999 |
| LGC354C | 5′-CCG AAG ATT CCC TAC TGC-3′ | 18 | *Firmicutes*[a] | Meier *et al.*, 1999 |

[1] http://www.microbial-ecology.net/probebase/

| Name | Sequence | Length (nt) | Specificity | Reference |
|---|---|---|---|---|
| DELTA495a | 5′-AGT TAG CCG GTG CTT CCT-3′ | 18 | Most *Deltaproteobacteria* and most *Gemmatimonadetes* | Loy *et al.*, 2002; Lücker *et al.*, 2007 |
| DELTA495b | 5′-AGT TAG CCG GCG CTT CCT-3′ | 18 | Some *Deltaproteobacteria* | Loy *et al.*, 2002; Lücker *et al.*, 2007 |
| DELTA495c | 5′-AAT TAG CCG GTG CTT CCT-3′ | 18 | Some *Deltaproteobacteria* | Loy *et al.*, 2002; Lücker *et al.*, 2007 |
| ARCH915 | 5′-GTG CTC CCC CGC CAA TTC CT-3′ | 20 | *Archaea* | Stahl & Amann, 1991 |

[a]Together, the three LGC354 probes target most of the *Firmicutes* but not *Desulfosporosinus* or *Desulfotomaculum* (Loy *et al.*, 2002).

## 2.4. Buffers, solutions, and other chemicals

All solutions were prepared using distilled, UV-light treated, filtered and deionized water (Milli-Q Water Purification System), with the exception of PCR reagents, where double distilled water was used. Chemicals were purchased in *pro analysis* or molecular biology grade.

### 2.4.1. Capillary electrophoresis

**Table 2.6** Alkalinization mix

| Name | Concentration (mmol $L^{-1}$) | Amount for 500 mL (g) |
|---|---|---|
| Sodium hydroxide (Carl Roth) | 500 | 10.0 |
| Sodium hexanoate (Sigma-Aldrich) | 4 | 0.2763 |
| Water | | fill up |

### 2.4.2. Agarose gel electrophoresis

Gels were prepared in a microwave with LE Agarose (Biozym) and 1× TBE buffer (Table 2.7). 6× DNA Loading Dye (Fermentas) was used to load samples. Applied DNA ladders are listed in Table 2.8. Gels were stained with ethidium bromide (Sigma-Aldrich) postrun.

**Table 2.7** 10× TBE buffer (pH 8.3).

| Name | Concentration (mmol $L^{-1}$) | Amount for 1 L (g) |
|---|---|---|
| Tris (Carl Roth) | 890 | 107.8 |
| Boric acid (Carl Roth) | 890 | 55.0 |
| EDTA disodium salt dihydrate (Carl Roth) | 20 | 7.4 |
| Water | | fill up |

**Table 2.8** DNA ladders by Fermentas.

| Name | Range (bp) |
|---|---|
| GeneRuler™ 1 kb DNA Ladder | 250–10000 |
| GeneRuler™ 50 bp DNA Ladder | 50–1000 |
| O'RangeRuler™ 50 bp DNA Ladder | 50–1000 |
| O'RangeRuler™ 20 bp DNA Ladder | 20–300 |

### 2.4.3. Nucleic acids extraction

The following chemicals were used for DNA and RNA extractions:

- Phenol/chloroform/isoamyl alcohol (PCI), 25:24:1 mixture, pH 5.2 ± 0.3 (Fisher Scientific).
- Chloroform/isoamyl alcohol (CI), 24:1 mixture (Carl Roth).
- Ethanol was purchased in HPLC Gradient Grade (Carl Roth) and undiluted, 70 %, and 75 % dilutions were prepared with DEPC-treated water in 50 mL reaction tubes.
- Glycogen, RNA grade, 20 mg $mL^{-1}$ (Fermentas).

Solutions were treated with 0.1 % (v/v) Diethylpyrocarbonate (DEPC; Sigma-Aldrich) to deactivate RNases and DNases (Blumberg, 1987). The mixture with DEPC was stirred overnight under a fume hood; the remaining DEPC was deactivated by autoclaving. Alternatively, if DEPC-treatment was not possible, chemicals were solved in DEPC-treated water.

**Table 2.9** CTAB/$KPO_4$ buffer (treated with DEPC). pH was adjusted to 8.0 with HCl and KOH (Carl Roth). For use in the CTAB-based extraction protocol.

| Name | Concentration | Amount for 250 mL (g) |
|---|---|---|
| $K_2HPO_4$ (Avantor) | 112.87 mmol $L^{-1}$ | 4.9 |
| $KH_2PO_4$ (Avantor) | 7.13 mmol $L^{-1}$ | 0.2426 |
| NaCl (Carl Roth) | 350 mmol $L^{-1}$ | 5.1 |
| CTAB (Carl Roth) | 5 % | 12.5 |
| DEPC-treated water | | fill up |

**Table 2.10** PEG 8000 solution (treated with DEPC). For use in the CTAB-based extraction protocol.

| Name | Concentration | Amount for 100 mL (g) |
|---|---|---|
| PEG 8000 (Sigma-Aldrich) | 30 % (w/v) | 30 |
| NaCl | 1.6 mol $L^{-1}$ | 9.4 |
| DEPC-treated water | | fill up |

**Table 2.11** Phosphate buffer. pH was adjusted to 8.0 with HCl (Carl Roth) and NaOH, and the solution was filter sterilized and autoclaved. For use in the TNS-based extraction protocol.

| Name | Concentration (mmol $L^{-1}$) | Amount for 200 mL (g) |
|---|---|---|
| $Na_2HPO_4{\cdot}2H_2O$ (Carl Roth) | 112.87 | 4.0 |
| $NaH_2PO_4{\cdot}H_2O$ (Carl Roth) | 7.12 | 0.1965 |
| DEPC-treated water | | fill up |

**Table 2.12** TNS solution. pH was adjusted to 8.0 with HCl, followed by autoclaving. For use in the TNS-based extraction protocol.

| Name | Concentration | Amount for 200 mL (g) |
|---|---|---|
| Tris | 0.5 mol $L^{-1}$ | 12.1 |
| NaCl | 0.1 mol $L^{-1}$ | 1.2 |
| SDS (Carl Roth) | 10 % (w/v) | 20.0 |
| DEPC-treated water | | fill up |

**Table 2.13** Potassium acetate solution (treated with DEPC). For use in the TNS-based extraction protocol.

| Name | Concentration (mol $L^{-1}$) | Amount for 500 mL (g) |
|---|---|---|
| Potassium acetate | 7.5 | 368.1 |
| DEPC-treated water | | fill up |

**Table 2.14** PEG 6000 solution (treated with DEPC). For use in the TNS-based extraction protocol.

| Name | Concentration | Amount for 500 mL (g) |
|---|---|---|
| PEG 6000 (Sigma-Aldrich) | 30 % (w/v) | 150 |
| NaCl | 1.6 mol $L^{-1}$ | 46.8 |
| DEPC-treated water | | fill up |

**Table 2.15** Sodium citrate solution for use in the TRIzol® reagent protocol.

| Name | Concentration | Amount for 250 mL |
|---|---|---|
| Trisodium citrate dihydrate (Carl Roth) | 100 mmol $L^{-1}$ | 7.4 g |
| Ethanol | 10 % | 25 mL |
| DEPC-treated water | | fill up |

**Table 2.16** Sodium acetate solution. pH was adjusted to 5.2 with HCl. For use in the purification step after RNase treatment.

| Name | Concentration (mol $L^{-1}$) | Amount for 250 mL (g) |
|---|---|---|
| Sodium acetate trihydrate (Carl Roth) | 3 | 102.1 |
| Water | | fill up |

### 2.4.4. Anoxic cultivation medium

Modified from Widdel & Bak (1992), see also Section 2.5.2.

**Table 2.17** Basal freshwater medium. Autoclaved.

| Name | Concentration (mmol $L^{-1}$) | Amount for 2 L (g) |
|---|---|---|
| Sodium chloride | 17.11 | 2.0 |
| Magnesium chloride hexahydrate (Carl Roth) | 1.97 | 0.8 |
| Monopotassium phosphate | 1.47 | 0.4 |
| Ammonium chloride (Carl Roth) | 4.67 | 0.5 |
| Potassium chloride (Merck) | 6.71 | 1.0 |
| Calcium chloride dihydrate (Avantor) | 0.68 | 0.2 |
| Water | | fill up |

**Table 2.18** Selenite-tungstate solution. Autoclaved.

| Name | Concentration (mmol $L^{-1}$) | Amount for 1 L (mg) |
|---|---|---|
| Sodium hydroxide | 10.00 | 400 |
| Sodium selenite pentahydrate | 0.02 | 6 |
| Sodium tungstate dihydrate | 0.02 | 8 |
| Water | | fill up |

**Table 2.19** Trace elements solution SL 12. pH was adjusted to 6.5 with NaOH, followed by autoclaving.

| Name | Concentration (mmol $L^{-1}$) | Amount for 1 L (g) |
|---|---|---|
| Iron(II) sulfate heptahydrate (Carl Roth) | 5.533 | 1.538 |
| Zinc chloride (Honeywell) | 0.514 | 0.070 |
| Manganese(II) chloride tetrahydrate (Carl Roth) | 0.505 | 0.100 |
| Cobalt(II) chloride hexahydrate (Sigma-Aldrich) | 0.799 | 0.190 |
| Copper(II) chloride dihydrate (Sigma-Aldrich) | 0.012 | 0.002 |
| Nickel(II) chloride hexahydrate (Honeywell) | 0.101 | 0.024 |
| Sodium molybdate dihydrate (Merck) | 0.074 | 0.018 |
| Boric acid | 4.852 | 0.300 |
| EDTA disodium salt dihydrate | 8.923 | 3.321 |
| Water | | fill up |

**Table 2.20** Vitamins solution. Filter sterilised and stored in the dark.

| Name | Concentration (µmol $L^{-1}$) | Amount for 200 mL (mg) |
|---|---|---|
| Vitamin $B_{12}$ | 37 | 10 |
| 4-Aminobenzoic acid | 365 | 10 |
| D(+)-Biotin | 41 | 2 |
| Nicotinic acid | 812 | 20 |
| Calcium D(+)-pantothenate | 52 | 5 |
| Pyridoxamine dihydrochloride | 622 | 30 |
| Thiamine hydrochloride | 148 | 10 |
| Water | | fill up |

## 2.5. Anoxic techniques

### 2.5.1. General

To mimic anoxic conditions as found in peatlands, all incubation and cultivation handling was done under an oxygen free atmosphere. Two different kinds of gases were used: (1) 100 % nitrogen gas ($N_2$, ≥ 99.999 % pure, Air Liquide) for all experiments where conditions as close to *in situ* as possible were required and (2) a mix of 80 % $N_2$ and 20 % $CO_2$ (≥ 99.9 % pure, Air Liquide) for cultivation experiments.

A technique to close vessels in a sterile and anoxic way was described before (Hungate, 1969). Under a constant gas stream, bottles and tubes were closed with black butyl rubber septa (Ochs) and clamped aluminium or screw caps. Rubber septa were prepared by washing in approximately 2 % oxalic acid solution, followed by repeatedly autoclaving in water until no discolouring was visible.

Preparation of anoxic solutions was done by repeatedly applying vacuum, adding pure $N_2$ or $N_2/CO_2$ gas and shaking to facilitate gas exchange (at least 3 repeats). Sterile syringes and needles (Braun) were used to transfer liquids and take samples. Flushing of syringes with anoxic gas prior to sampling ensured minimal introduction of oxygen to running experiments.

### 2.5.2. Preparation of cultivation medium

For enrichment and cultivation experiments, anoxic freshwater medium modified from Widdel & Bak (1992) was prepared. The basal medium (Table 2.17) was removed from the autoclave at 75 °C and immediately attached to a constant $N_2/CO_2$ stream. After the medium was cooled down, 2 mL of each of

the trace elements solution SL 12 (Table 2.19), the selenite-tungstate solution (Table 2.18) and the vitamins solution (Table 2.20) were added. Additionally, anoxic sodium bicarbonate solution was added (60 mmol sodium bicarbonate dissolved in approximately 60 mL of water; Merck), followed by adjusting the pH to approximately 7.1 with hydrochloric acid (1 mol $L^{-1}$). The finished medium was aliquoted in bottles sealed with rubber septa. A few crystals sodium dithionite (Honeywell) were added to the finished medium and all medium aliquots.

## 2.6. Anoxic incubations

### 2.6.1. Long-term soil slurry incubations

A soil core from Schlöppnerbrunnen II (sampling site A III; Figure 2.1) was extracted in September 2010 by Dr. Michael Pester and Mag. Norbert Bittner (Department of Microbial Ecology). From this soil core, the depth 10–20 cm was selected for use in this incubation experiment. Furthermore, several litres of fen water were taken from a surface pool at the sampling site (Figure 2.1d). The samples were transported to Vienna and stored at 4 °C until the start of the experiment.

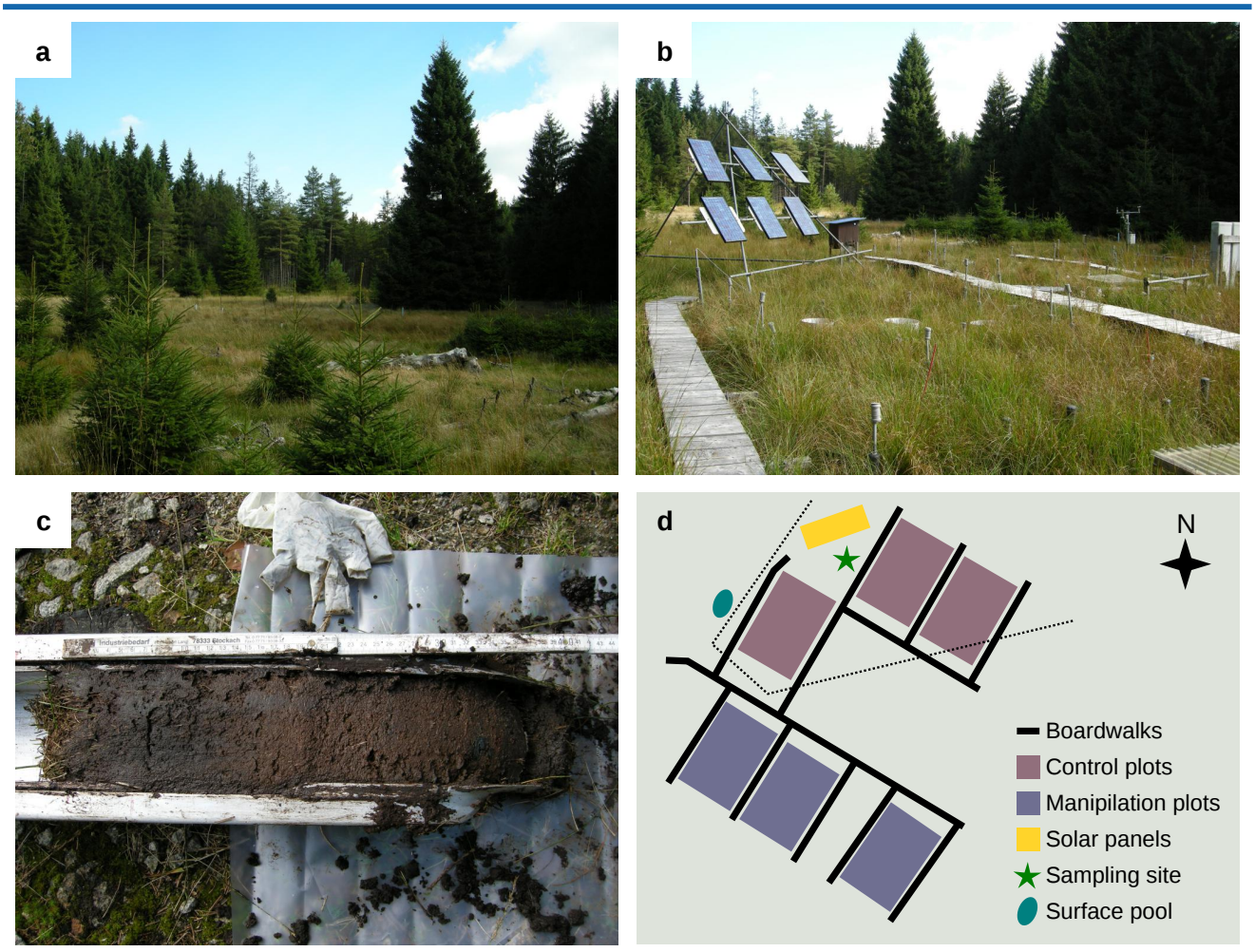


**Figure 2.1** Photos of the Schlöppnerbrunnen II fen; taken while sampling in September 2010 by Dr. Michael Pester and Mag. Norbert Bittner. **(a)** Plant vegetation. **(b)** Test plant of the University of Bayreuth. **(c)** Freshly sampled soil core. **(d)** Schematic plan of the test plant. Dotted lines indicate the viewpoint of the photo (b). Control and manipulations plots used for biogeochemical studies (e.g. Knorr *et al.*, 2009; Reiche *et al.*, 2009).

Consecutive sterile filtration was done with the sampled peatland water (5.00 µm, 0.45 µm and finally 0.2 µm filters). The last filtration step was done by inserting the filter (plus a needle) directly into a

sealed, sterile bottle (Schott) and applying constant underpressure to the bottle with a vacuum pump. The remaining oxygen was replaced with $N_2$ by above described methods. In an anoxic box filled with $N_2$, soil slurries were prepared in 250 mL glass bottles (Schott). For each microcosm 60 mL of sterile and anoxic fen water were added to 30 g of soil and microcosms were sealed under anoxic conditions with butyl rubber septa.

For analysis of time point zero, approximately 30 g of soil were frozen at −80 °C in two separate 50 mL reaction tubes. Another 30 g of soil were mixed with 60 mL of fen water to measure the starting pH of such an incubation.

During the whole experiment, the slurries were kept in the dark at 14 °C and only removed shortly for adding substrates or sampling (at room temperature). The general procedure consisted of three parts, which were repeated for eight weeks (an exact incubation schedule can be found in Table A.2):

*Adding sulfate and substrates*
: Syringes and needles flushed with nitrogen gas were used to take up anoxic sulfate/substrate, add it to the microcosm, and take out the same amount of gas phase to avoid overpressure (Figure 2.2b, c). Following the amendment, the bottles were shaken and put pack on 14 °C. Frequency and approximate amount of sulfate and substrate addition is described in Table 2.21.

*Taking liquid samples*
: After addition of sulfate/substrates, approximately 300 µL of the liquid phase from the soil slurries were sampled (Figure 2.2c), intermediately put on ice and then frozen at −20 °C. These samples were used for analysis with capillary electrophoresis.

*Taking soil samples*
: Soil samples were taken from the slurries the day after sulfate/substrate addition. No soil samples were taken after days where only formate was added. In weeks 4–8 sampling was reduced to once per week; sampling at day 20 was moved to day 21 because of time constraints. Under constant $N_2$ stream, the bottles were opened and a sterile 1 mL pipette tip with a cut-off end was used to sample approximately 1.5 mL of soil (Figure 2.2d). The soil was immediately flash frozen in liquid nitrogen and stored at −20 °C and later at −80 °C. The bottles were closed again with a fresh $N_2$ atmosphere. This also served as an aeration step to simulate the removal of gases like $CH_4$ or $H_2S$ by diffusion.

**Table 2.21** Addition schedule of *in situ* sulfate and substrate concentrations to soil slurries. A more detailed schedule is given in Table A.2.

| Substrate | Stock concentration (mmol $L^{-1}$) | Amount[a] | Weekly supply |
|---|---|---|---|
| Sodium formate (Sigma-Aldrich) | 24 | 300 (120) | 3–4×[b] |
| Sodium acetate (Carl Roth) | 24 | 300 (120) | 2× |
| Sodium propionate (Sigma-Aldrich) | 24 | 300 (120) | 2× |
| Sodium L-lactate (Sigma-Aldrich) | 24 | 300 (120) | 2× |
| Sodium butyrate (Sigma-Aldrich) | 24 | 300 (120) | 2× |
| Sodium sulfate (Merck) | 48 | 130 (104)[c] | 1–2×[d] |

[a]Values given are amounts added (µL) and final concentrations in parentheses (µmol $L^{-1}$, assumed total volume: 60 mL).

[b]Due to its low energy yield, formate addition was raised to 4 times per week from week 4 to 8.

[c]250 µL (200 µmol $L^{-1}$) of sulfate were added on the first day of sampling instead of 130 µL (104 µmol $L^{-1}$), to stimulate sulfate reduction.

[d]After supplying larger amounts in the first week (250 µL and 130 µL), sulfate addition was reduced to once per week for 3 weeks and then raised again to twice per week from week 5 to 8.

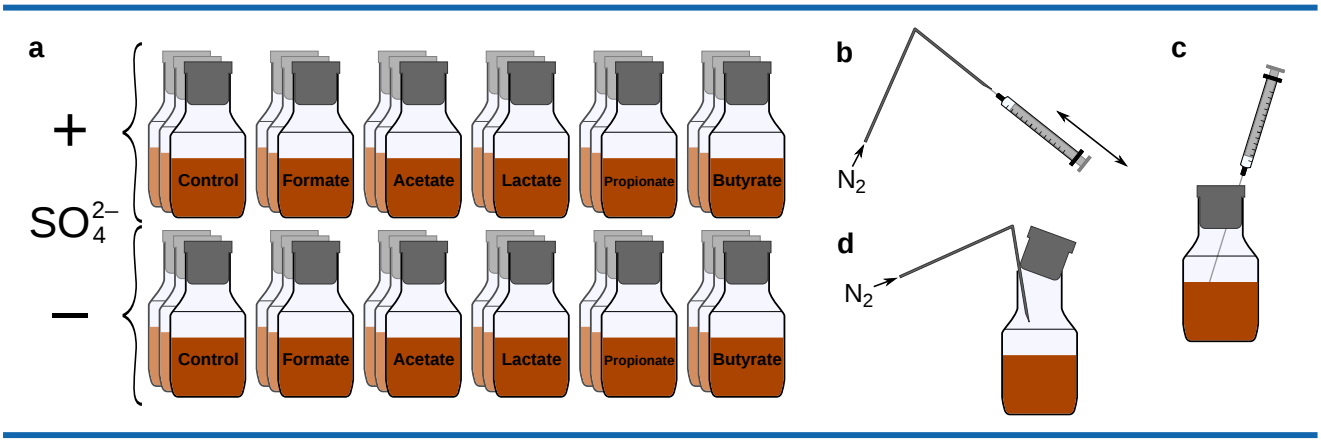


**Figure 2.2** **(a)** 36 soil slurry microcosms were used for this incubation experiment. Each different substrate scenario was done in biological replicates. **(b)** Syringes and needles were flushed with anoxic gas by repeatedly pushing in and pulling out the plunger while inserted into a bigger needle connected to an $N_2$ stream. **(c)** Adding sulfate/substrates or taking liquid samples was done by injecting a needle through the rubber stopper. In advance the rubber stopper was sterilized by wiping it with 70 % ethanol or flaming. **(d)** A bent needle, connected to a $N_2$ stream, was inserted into an open microcosm to keep it anoxic during soil sampling.

Only one substrate was added per microcosm (six microcosms per substrate). Out of these six slurries, three were also regularly amended with sulfate (Table 2.21). Furthermore, as controls, three microcosms were incubated without any amendment and three more were supplemented only with sulfate (Figure 2.2a, Table B.1).

On day 54, two microcosms of each triplicate set were completely sampled. The remaining slurry was poured into two 50 mL reaction tubes and then centrifuged at 2 °C (5 min, 6 000 × *g*). The supernatant was removed and used for pH measurements and the remaining soil pellet was flash frozen in liquid nitrogen and stored at −80 °C. The last replicate was kept at 14 °C for use in the enrichment experiment.

### 2.6.2. Short-term soil slurry incubations

Similar to the long-term experiment, a six day incubation was set up by Dr. Michael Pester and Dr. Klaus-Holger Knorr (University of Bayreuth) at the University of Bayreuth (detailed in Table B.2). Sulfate and the same substrates at the same concentrations as in the long-term incubations were added at days 0 and 5 of the experiment (200 and 104 µmol $L^{-1}$ of sulfate, and 120 and 120 µmol $L^{-1}$ of substrates, respectively). Formate was also added on day 3. Liquid samples were taken on every day. They were analysed with capillary electrophoresis by the author of this document. On day 3 and 5 sampling was done before sulfate/substrate addition. $^{35}S$ radiotracer, as well as gas chromatography (GC) measurements were done by Dr. Knorr to determine sulfate reduction rates and concentrations of $CH_4$, $CO_2$ and $H_2$ (GC data will not be presented in this document).

### 2.6.3. Enrichment cultures

One sulfate-amended microcosm per substrate (from the long-term incubations), including a sulfate-amended control, was further incubated at 14 °C with 5 mmol $L^{-1}$ of sulfate and 4 mmol $L^{-1}$ of appropriate substrates (in case of the formate slurry: 10 mmol $L^{-1}$ and 2.5 mmol $L^{-1}$ of sulfate). 27 days later the same amount of sulfate and substrates was added once more. Another 47 days later, 2 mL of each slurry were transferred to tubes with 8 mL defined anoxic cultivation medium (Section 2.5.2). Again 5 mmol $L^{-1}$ of sulfate were added. Substrates were added in concentrations that corresponded to 100 % mineralisation to $CO_2$ by sulfate reduction (20 mmol $L^{-1}$ formate, 5 mmol $L^{-1}$ acetate, 3.3 mmol $L^{-1}$ lactate, 2.9 mmol $L^{-1}$ propionate and 2.0 mmol $L^{-1}$ butyrate; see also Table 1.2). In case of the control

microcosm, only 1 mL slurry was transferred and approximately 1 mL of sterilised Schlöppnerbrunnen soil was added as a source of substrates.

Sulfate and substrate turnover and microbial growth were monitored via capillary electrophoresis and optical density measurements at 600 nm, respectively. When sulfate turnover or growth was detected, approximately 0.1–1.0 mL of the enrichment culture were used to inoculate a tube with fresh medium, sulfate, and substrate (total volume approximately 10 mL). This enrichment step was done to outdilute soil particles and non- or slow-growing microorganisms, and to avoid depletion of essential medium components such as vitamins. Depending on the current sulfate and substrate levels of an enrichment step, additional sulfate/substrate was added and the tube was incubated for a few more days before inoculation in fresh medium. The purpose of this step was to increase the chance of transferring SRMs which are in the exponential growth phase.

The inoculation of fresh medium tubes was repeated multiple times until a stable enrichment culture was growing. Non-growing enrichment cultures were also amended with 10 mg $L^{-1}$ of yeast extract (Oxoid) to compensate for missing traces elements.

## 2.7. Capillary electrophoresis

Since sulfate and all substrates are negatively charged in solution, a capillary electrophoresis machine was used for quantification (Beckman Coulter P/ACE™ MDQ Molecular Characterization System). The principle of separation and detection of negatively charged small molecules via capillary electrophoresis is shown in Figure 2.3. The sample is injected into the capillary by applying overpressure to the sample vial, then a high power electric field is activated (30 kV). The molecules migrate at different speeds through the capillary, depending on their charge and size, and can be individually detected near the positive pole by UV light absorption.

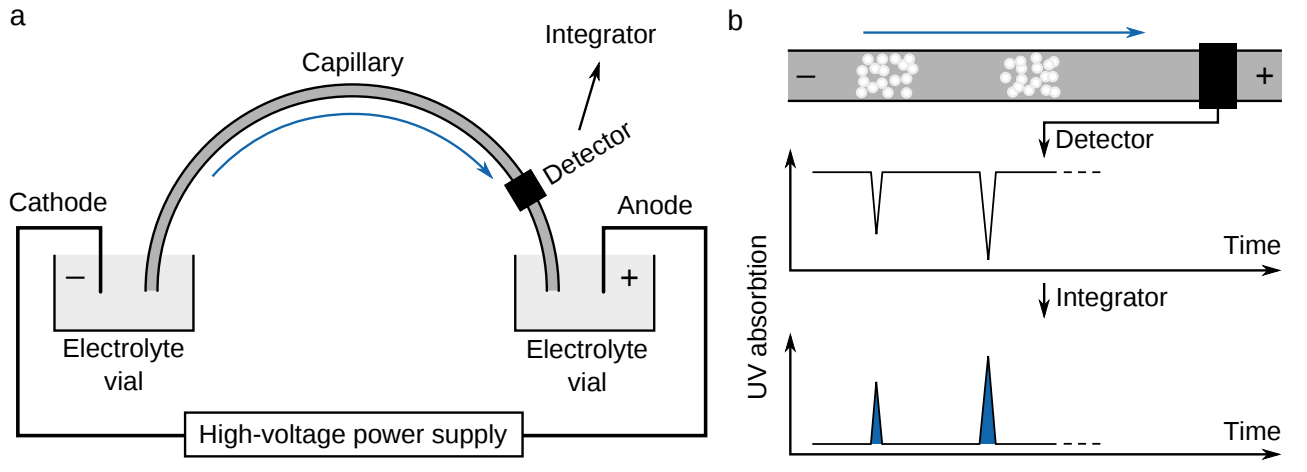


**Figure 2.3** A capillary electrophoresis system (based on Skoog *et al.*, 2007 and the CEofix™ Anions 5 kit manual). **(a)** After the sample is injected, the capillary is inserted into the electrolyte buffer and the power supply is activated. The blue arrow indicates the direction of the anion migration. **(b)** Anions from the sample (white dots) displace the electrolyte buffer (grey). The detector measures drops in UV absorption, which are then inverted and integrated (blue areas).

Since the target compounds are mostly UV transparent, a kit designed to indirectly detected small anions and organic acids was used (Analis CEofix™ Anions 5 kit). The background electrolyte from this kit contains an UV absorbing substance, which is displaced by sulfate/substrates. This absence of UV absorption can be detected by the capillary electrophoresis machine. The signal is then inverted

*in silico* and plotted (Figure 2.3b). The kit was used according to the manufacturers recommendations with the following modifications: injection time was changed from 8 to 20 seconds for increased sample uptake and therefore increased sensitivity; cartridge temperature was set to 20 °C.

An approximately 65 cm long untreated fused-silica capillary with an inner diameter or 75 µm was used. Capillaries from two different suppliers were used: (1) Beckman Coulter eCAP and (2) Polymicro. The Beckman Coulter capillary turned out to be very fragile so the Polymicro capillary was used for most of the measurements.

Quantification and identification of detected peaks was done by comparison to defined standard mixtures. These consisted of equimolar amounts of sodium sulfate, formate, acetate, lactate, propionate and butyrate at the following concentrations: 6.25, 12.5, 25, 50, 100, 200, 400, 800 and 1600 µmol $L^{-1}$. The lowest and the two highest standards were only used if required. During one measurement session (normally one whole day) the calibration mixes were scanned once at the beginning and then the order of samples and a second run of all standards was randomly mixed to reduce technical biases.

Standard and sample preparation protocol:

- Thaw on ice.
- Vortex briefly.
- Centrifuge at full speed for at least 5 min at 2 °C.
- Mix 60 µL of supernatant with 3 µL of alkalinization mix (Table 2.6).

Measurements were started immediately after alkalinization and 6–9 samples where prepared per capillary electrophoresis run. With the P/ACE™ MDQ Molecular Characterization System samples are measured in series and the analysis time per sample with the CEofix™ Anions 5 kit is 15 minutes, meaning that the ninth sample would then be analysed after two hours. Measuring samples later than two hours after preparation would results in diminished precision (Dr. Michael Pester, unpublished data) and was therefore avoided.

The alkalinization mix contained sodium hexanoate as an internal standard (final concentration in mixture with sample: 190 µmol $L^{-1}$). Using an internal standard is advantageous and necessary, because: (1) The shape of the hexanoate peak is an indicator for the quality of a scan. (2a) Although the sample injection method was always the same, the machine can not guarantee that the injected sample volume stays the same between all scans. The hexanoate concentration on the other hand was always the same and its peak area was used to normalise the sulfate/substrate peaks. (2b) Additionally, the peak retention times are shifting over the course of one measurement session. Again, the retention times of sulfate and substrate peaks can be normalised against hexanoate, which simplifies peak identification.

Integration of all traces was done with the 32 Karat software (Beckman Coulter) which is bundled with the P/ACE™ MDQ Molecular Characterization System. The automatic annotation feature was used for preliminary integration followed by manual proof reading and, if necessary, correction of peak starts, ends and baselines.

## 2.8. Nucleic acids extraction

Two different types of samples were used for evaluation of DNA and RNA isolation and preparation: (1) Enrichment microcosm 25 was amended with butyrate and sulfate, the $N_2$ atmosphere was refreshed and 300 µL test samples were taken within a few days. (2) Soil from time point zero was ground in liquid nitrogen and approximately 300 mg were used as test samples.

### 2.8.1. Phenol-based nucleic acids extraction methods

Most samples were already frozen in lysis matrix tubes (MP Biomedicals). Samples frozen in normal reaction tubes were transferred with flamed spatulas. The wet/frozen weight of all samples was determined prior to extraction by measuring the empty and filled sample tubes (Equation 2.1).

**Equation 2.1** Indirect determination of sample weight.

$$\text{weight}_{\text{sample}} = \text{weight}_{\text{tube + sample}} - \text{weight}_{\text{empty tube}}$$

Two methods, based on cell lysis by bead beating and phenol/chloroform/isoamyl alcohol (PCI) extraction, were evaluated:

*TNS-based extraction*
: This protocol is based on Lueders *et al.* (2004). An additional step of humic acids precipitation with potassium acetate (Bodrossy *et al.*, 2006) was added. A detailed protocol is listed in Section A.1.

*CTAB-based extraction*
: This protocol is based on Griffiths *et al.* (2000) with modifications from Leininger *et al.* (2006). A detailed protocol is listed in Section A.2.

### 2.8.2. Grinding in liquid nitrogen prior to nucleic acids extraction

Samples were transferred from the −80 °C freezer to a −20 °C freezer or dry ice, where they were kept until processed. Mortar and pestle were cleaned with water, 70 % and 96 % ethanol. The ethanol remaining in the mortar was then ignited. Mortar and pestle were optionally pre-cooled in a −20 °C freezer (covered with a clean autoclave bag). The Mortar was cooled with liquid nitrogen until the evaporation was minimal, then the sample was added. The soil samples were ground to a fine powder. Enough liquid nitrogen always remained in the mortar so that the sample did not thaw. Grated samples were transferred to pre-weighted Lysing Matrix tubes, were weighted again using an analytical balance and stored at −80 °C or directly applied the nucleic acids extraction. A step-by-step protocol is given in Section A.3.

### 2.8.3. Methods for separation and purification of DNA and RNA

*AllPrep DNA/RNA Mini Kit*
: This kit by QIAGEN uses spin columns, which specifically bind genomic DNA and separate it from the RNA. Additionally, both the DNA and RNA aliquots are purified with on-column wash steps. The kit was used according to the manufacturers recommendations, with the following exception: since cell homogenisation was already done with the phenol-based protocols, the lysed samples were mixed with 650 µL of buffer RLT Plus and 6.5 µL of β-mercaptoethanol (14.3 mol $L^{-1}$, Sigma-Aldrich) and then applied to the first column. Additional on-column DNase I treatment with the QIAGEN RNase-Free DNase Set was also tested.

*TRIzol® reagent*
: The TRIzol® reagent (Invitrogen) is a commercial version of the phenol-based extraction method and is designed to isolate DNA, RNA, and proteins from one sample. Homogenisation of soil samples was done with the above descried bead-beating and PCI-based methods. 1 mL of the TRIzol® reagent was added to the total nucleic acids extract and the manufacturers protocol was followed starting from the “Phase separation” step.

*Lithium chloride precipitation*
RNA can be selectively precipitated by lithium chloride and therefore separated from DNA. This method was done according to the protocol of Untergasser (2008), where RNA is precipitated and washed in one tube and the supernatant (DNA) is transferred to a new tube. This means that traces of DNA may remain in the original tube, therefore a combination of lithium chloride precipitation with DNase treatment of the RNA aliquot was also evaluated.

*Direct DNase digestion*
For DNase I treatment of nucleic acids samples the TURBO DNA-free™ Kit (Applied Biosystems) was used according to the manufacturers recommendations. A slightly adapted step-by-step protocol is listed in Section A.5. This kit efficiently removes the DNase and also divalent cations after the digestion, so no further steps are required before reverse transcription (RT).

*Direct RNase digestion*
RNA was removed from DNA aliquots with the RNase ONE Ribonuclease (Promega) by following the manufacturers recommendations and the RNase I treatment protocol[2] from the U.S. Department of Energy Joint Genome Institute. The protocol includes the digestion as well as the DNA de-salting steps (Section A.6; Sambrook & Russell, 2001).

*OneStep™ PCR Inhibitor Removal Kit*
The OneStep™ PCR Inhibitor Removal Kit by Zymo Research is a spin column based nucleic acids purification system. It is especially designed to remove PCR inhibitors, for example polyphenolics, humic/fulvic acids, tannins and/or melanin[3]. A step-by-step protocol based on the manufacturers manual is detailed in Section A.4.

## 2.9. Nucleic acids quantification

DNA and RNA samples were quantified with the Quant-iT™ PicoGreen® dsDNA Reagent and Kit, and Quant-iT™ RiboGreen® RNA Reagent and Kit, respectively. The kits were used according to the manufactures recommendations, with the following modifications. The manuals recommend total reaction volumes of 1 mL and the usage of cuvettes. Researchers at the Department of Microbial Ecology determined that it was possible to downscale the assays to 100 and 200 µL reaction volumes for accurate DNA and RNA quantification, respectively. The assays were prepared in black 96-well-microplates (Greiner Bio-One), followed by measurement with the Infinite M200 microplate reader.

Samples were measured in triplicates (unless stated otherwise), while standards were measured in duplicates. The Infinite M200 microplate reader measures row by row, so the standard curve solutions were mixed in the second and next-to-last rows of the plate, to reduce bias caused by the measurement delays. 50 µL of PicoGreen® dsDNA Reagent/100 µL of RiboGreen® RNA Reagent were added last and in the same step the wells were mixed by pipetting. The plate was covered with PCR film and aluminium foil, spun down and measured (without cover, automatic optimal gain).

The range of the standard dilution series was extended to 0, 20, 40, 80, 160, 200, 400, 600, 800, and 1000 ng $mL^{-1}$ in the case of DNA and 0, 1, 2, 4, 8, 10, 20, 30, 40, and 50 ng $mL^{-1}$ final in-well concentration in the case of RNA quantification. Sample aliquots were diluted with 1× TE buffer to be within the range of the standard curve.

[2]http://my.jgi.doe.gov/general/protocols/RNase_I_DNA_Prep_Clean_up_protocol.doc
[3]http://www.zymoresearch.com/rna-purification/rna-clean-up/rt-inhibitor-removal/onestep-pcr-inhibitor-removal-kit

## 2.10. Standard and quantitative real-time PCR

### 2.10.1. General

The qPCR assays for quantification of *Desulfosporosinus* species and total *Bacteria & Archaea* were already established, optimised and evaluated at the Department of Microbial Ecology (Pester *et al.*, 2010). The chosen kit was the Platinum® SYBR® Green qPCR SuperMix-UDG from Invitrogen, which uses the SYBR Green I dye. For enhanced performance and repeatability this kit is premixed and only primers, the template and additional (optional) components have to be added (Table 2.22 and Table 2.23).

If not stated otherwise, samples were analysed in technical triplicates and standard curves were done in technical duplicates. Controls were done in every assay. 1–3 replicates of no-template controls (NTC; 5 µL of PCR water) were always included. A no-target control was also included in the *Desulfosporosinus*-sepcific qPCR assay. For this purpose the Schlöppnerbrunnen 16S rRNA gene clone SII-2-102 from a previous study (Pester *et al.*, 2010; GenBank accession number: GU270795.2) was selected, which is an unclassified *Acidaminococcaceae* (*Firmicutes*).

**Table 2.22** Components in one qPCR reaction targeting total *Bacteria & Archaea* or *Desulfosporosinus*.

| Reagent | *Bacteria & Archaea* (µL) | *Desulfosporosinus* (µL) |
|---|---|---|
| 2× SuperMix (Invitrogen) | 25.00 | 25.00 |
| Fluorescein Calibration Dye (1 µmol $L^{-1}$, Bio-Rad) | 0.50 | 0.50 |
| BSA (1 mg $mL^{-1}$, Invitrogen) | 0.25 | 0.25 |
| Primer 1389Fmix[a] (50 pmol $\mu L^{-1}$) | 0.75 | 0.00 |
| Primer 1492R (50 pmol $\mu L^{-1}$) | 1.00 | 0.00 |
| Primer DSP603F (50 pmol $\mu L^{-1}$) | 0.00 | 1.00 |
| Primer DSP821R (50 pmol $\mu L^{-1}$) | 0.00 | 0.75 |
| Template DNA or cDNA[b] | 5.00 | 5.00 |
| PCR water | 17.50 | 17.50 |

[a]In early analysis the primer 1389Farch was used instead.

[b]In the final protocol, the concentration of DNA samples was 5 ng $\mu L^{-1}$ and the cDNA was diluted 10-fold before it was applied (RNA concentration before reverse transcription was 1 ng $\mu L^{-1}$). DNA and cDNA amounts varied during the evaluation steps.

**Table 2.23** qPCR thermal cycling program.

| Repeats | Temperature (°C) | Time (min) | Note |
|---|---|---|---|
| 1× | 95 | 05:00 | |
| 40–45× | 95 | 00:40 | |
| | 52/64[a] | 00:30 | |
| | 72 | 00:40 | real time detection |
| 1× | 95 | 01:00 | |
| 1× | 55 | 01:00 | |
| 80× | 55 | 00:10 | raise temperature by 0.5°C every step (melt curve) |
| 1× | 4 (20) | ∞ | hold (over night) |

[a]52 °C or 64 °C were used for qPCR assays targeting total *Bacteria & Archaea* or *Desulfosporosinus*, respectively

qPCR standard aliquots for both assays were freshly prepared at the beginning of this diploma thesis. DNA extracts of the *Desulfosporosinus* 16S rRNA gene clone SII-2-12 and a *Syntrophobacter wolinii* 16S rRNA gene clone were already available at the Department of Microbial Ecology (see Appendix C). PCR with M13 Forward and M13 Reverse primers was performed (Table 2.24 and Table 2.25) and controlled with agarose gel electrophoresis (1 % agarose, 120 V, 60 min). PCR products were cleaned with the QIAquick PCR Purification Kit according to the manufactures instructions and eluted in 30 µL of PCR water.

Quantification of PCR products was done using the Quant-iT™ PicoGreen® dsDNA Reagent and Kit. A dilution series of both 16S rRNA gene standards was produced and aliquots were stored at −20°C. The established qPCR assays were used to verify the quality of the dilution series. The *S. wolinii* based 16S rRNA gene dilution series was used as the standard for quantification of total *Bacteria* & *Archaea* 16S rRNA gene copy numbers. Calculated copy numbers of the standard dilutions series are given in Section A.8. PCR product lengths of the total *Bacteria* & *Archaea* standard amplified with the 1389F/1492R primers and the *Desulfosporosinus* standard amplified with the DSP603F/DSP821R primers are 123 bp and 236 bp, respectively.

*In silico* normalisation of copy numbers against template concentrations was done. Results derived from DNA samples were divided by the amount of applied DNA (5 ng in case of the incubation samples, varying amounts in the evaluation steps) to calculate the number of 16S rRNA gene copies in 1 ng of genomic DNA. Measured 16S rRNA copy numbers were also normalised against the amount of RNA sample that was applied to the reverse transcription and the amount of cDNA which was applied to the qPCR (1 ng RNA was reverse transcribed and 5 µL of a 10-fold cDNA dilution was quantified in case of the incubation samples, varying amounts and dilutions in the evaluation steps). However, the normalised copy numbers do not represent the number of 16S rRNA transcripts in 1 ng of RNA, since a reverse transcription of every single RNA molecules only once is unrealistic.

**Table 2.24** Components in one M13-PCR reaction.

| Reagent | Amount (µL) |
|---|---|
| 10× *Taq* Buffer with KCl (Fermentas) | 5.00 |
| dNTP Mix (2 mmol $L^{-1}$ each, Fermentas) | 5.00 |
| $MgCl_2$ (25 mmol $L^{-1}$, Fermentas) | 4.00 |
| Primer M13 Forward (50 pmol $\mu L^{-1}$) | 1.00 |
| Primer M13 Reverse (50 pmol $\mu L^{-1}$) | 1.00 |
| *Taq* DNA polymerase (recombinant, 5 u $\mu l^{-1}$, Fermentas) | 0.20 |
| Template DNA | 1.00–3.00 |
| PCR water | 32.80–30.80 |

**Table 2.25** M13-PCR thermal cycling program.

| Repeats | Temperature (°C) | Time (min) | Note |
|---|---|---|---|
| 1× | 95 | ∞ | hot-start |
| 1× | 95 | 03:00 | |
| 30× | 95 | 00:40 | |
| | 60 | 00:40 | |
| | 72 | 01:20 | |
| 1× | 72 | 10:00 | |
| 1× | 4 | ∞ | hold |

### 2.10.2. Testing for inhibition

PCR and qPCR (as well as reverse transcription) can be inhibited by numerous exogenous or endogenous inhibitors. To test for this unwanted effect, dilution series of selected samples were analysed with qPCR. Good linear correlation between applied sample amounts and copy numbers means that the reaction is not inhibited. If a qPCR assay is inhibited, no linear increase or even decrease in copy numbers should be observed at rising samples amounts. Dilution series were limited by the starting concentrations of the samples and the detection range of the assay.

Dilution series were plotted in two different ways: (1) Absolute copy numbers were plotted against sample amounts to analyse the linear regression. (2) Copy numbers were normalised to 1 ng of DNA or 1 ng of reverse transcribed RNA and then plotted against sample amounts. When normalised copy numbers were similar between each sample dilution, it could be inferred that no inhibitors were present.

## 2.11. Working with RNA

### 2.11.1. General guidelines

When working with RNA, it is important to minimize the introduction of RNases, since they are ubiquitous and very stable (Nielsen, 2011). Glassware was baked at 180 °C for 4–6 hours and all consumables were purchased free of RNases. Solutions were treated with DEPC, as described in Section 2.4.3, or RNase-free chemicals were solved in DEPC-treated water.

A work bench dedicated only to work with RNA is established at the Department of Microbial Ecology. All surfaces and pipettes were cleaned with RNase AWAY (Carl Roth). Work steps were done as quickly as possible and samples were kept on ice. Thaw-freeze cycles were kept to a minimum.

### 2.11.2. Endogenous DNA contamination in RNA samples

To test for remaining amounts of DNA in RNA samples (after separation of DNA and RNA), PCR was done as described in Table 2.26 and Table 2.27 and visualised with gel electrophoresis, or 5 µL of diluted RNA sample was applied to the already described qPCR assay.

**Table 2.26** Components in one PCR reaction used for endogenous DNA contamination testing.

| Reagent | Amount (µL) |
|---|---|
| 10× *Taq* Buffer with KCl | 5.00 |
| dNTP Mix (2 mmol $L^{-1}$ each) | 5.00 |
| $MgCl_2$ (25 mmol $L^{-1}$) | 4.00 |
| BSA (1 mg $mL^{-1}$) | 0.25 |
| Primer 1389Farch (50 pmol $\mu L^{-1}$) | 0.75 |
| Primer 1492R (50 pmol $\mu L^{-1}$) | 1.00 |
| *Taq* DNA polymerase (recombinant, 5 u $\mu l^{-1}$) | 0.25 |
| RNA | 1.00 |
| PCR water | 32.75 |

**Table 2.27** PCR thermal cycling program used for endogenous DNA contamination testing.

| Repeats | Temperature (°C) | Time (min) | Note |
|---|---|---|---|
| 1× | 95 | ∞ | hot-start |
| 1× | 95 | 05:00 | |
| 35× | 95 | 00:40 | |
| | 52 | 00:30 | |
| | 72 | 00:40 | |
| 1× | 4 | ∞ | hold |

### 2.11.3. Reverse transcription of RNA

Two reverse transcription kits by Invitrogen were used (Table 2.3) according to their recommended protocols. For both kits the random hexamers priming method was chosen to minimize selection of specific types of RNAs. The alternative methods in reverse transcription are specific or oligo(dT) primers.

To control the kit for contaminations, a negative control with DEPC-treated water was done for every batch of reverse transcriptions. Selected samples were additionally applied to reverse transcription without the reverse transcriptase enzyme in the reaction mix. The resulting solution was then applied to a 16S rRNA qPCR assay targeting total *Bacteria* & *Archaea* to test for detectable traces of DNA in the RNA aliquot.

## 2.12. Fluorescence *in situ* hybridization

rRNA-targeted fluorescence *in situ* hybridization (FISH) and samples fixation with paraformaldehyde (PFA) and ethanol was previously described (Daims *et al.*, 2005). Probes are listed in Section 2.3.

Some samples were stained, in addition, with 4′,6-Diamidino-2-phenylindole (DAPI; Carl Roth). A 1:1000 diluted DAPI solution was pipetted on sample wells, followed by a 10 min incubation in the dark and washing with water.

# Chapter 3. Results



## 3.1. Evaluation of nucleic acids extraction

Two key elements in any successful qPCR analysis are the quality and quantity of the extracted nucleic acids (Fleige & Pfaffl, 2006; Saleh-Lakha *et al.*, 2011). Both depend strongly on the chosen extraction protocol, which requires thorough testing of all methods. Nucleic acids quality was controlled by testing for exogenous or endogenous contaminations and inhibitors of reverse transcription or qPCR, and integrity of DNA and RNA was assessed at each evaluation step. In addition, increases in nucleic acids yields were required to gain sufficient DNA and RNA amounts for quality control steps and to be able to perform multiple qPCR analysis (Figure 3.1). The final extraction pipeline is shown in Figure A.1.

The extraction protocols were continuously fine-tuned during this study. However, when comparing different methods within one step of the evaluation process, all test samples were extracted and handled the same way. Two types of test samples were used: (1) In the long-term incubation experiment, one microcosm per substrate scenario was kept after the final sampling time point. Out of this 12 microcosms, the butyrate- and sulfate-amended microcosm (number 25) was selected as a source of test soil slurry samples, because considerable turnover of sulfate was observed (see Section 3.2). (2) Sufficient amounts of time point zero soil were sampled and could therefore be used for tests.

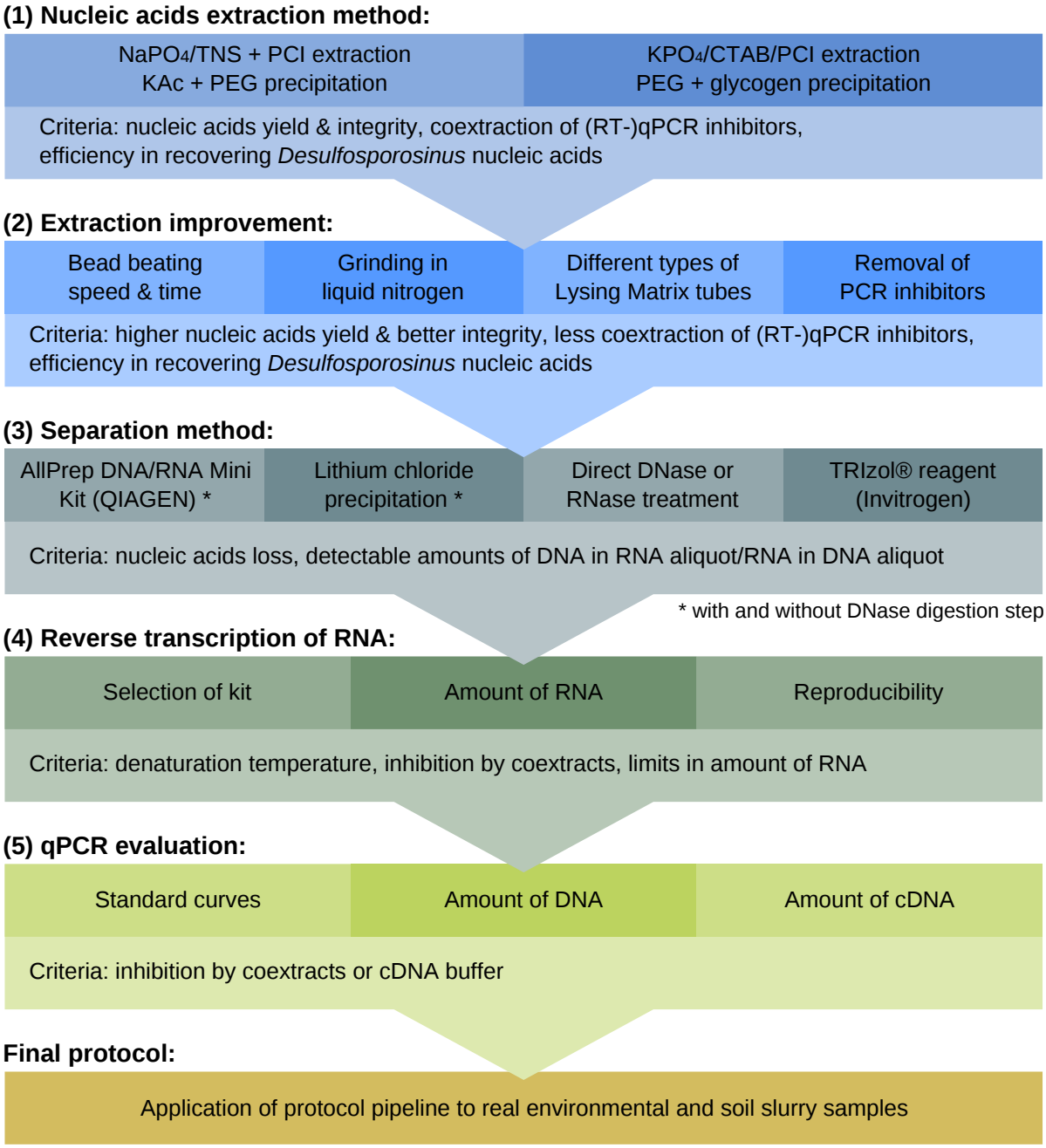


**Figure 3.1** Overview of all evaluated steps and methods for extracting nucleic acids from Schlöppnerbrunnen soil and analysis thereof with 16S rRNA qPCR assays. *PCI* phenol/chloroform/isoamyl alcohol; *KAc* potassium acetate.

### 3.1.1. Nucleic acids extraction methods

Two different phenol-based nucleic acids extraction protocols were compared: (1) the TNS-based method was previously used for a stable isotope probing study at the authors department (Pester *et al.*, 2010) and (2) the CTAB-based method was recommended by a cooperating department (Department of Genetics in Ecology). With the CTAB-based method, different bead beating parameters were tested (Table 3.1). Test samples from microcosm 25 were extracted with the TNS- and CTAB-based extraction protocols, but in contrast to the standard methods, nucleic acids pellets were resuspended in 50 µL DEPC-treated water (an early version of the CTAB-based protocol was used, with shorter centrifugation times and without addition of glycogen, Section A.2). After the potassium acetate precipitation step in

the TNS-based method only two thirds of the supernatant could be used in the next step because of the reaction tube sizes. The remaining supernatant of test samples 9 and 10 were combined and nucleic acids were also extracted following the TNS-based protocol starting with step 12 (see Section A.1) and labelled sample 11.

**Table 3.1** Microcosm 25 soil samples used for the evaluation of different nucleic acids extraction methods.

| Number | Method | Bead beating speed (m $s^{-1}$) | Bead beating time (s) |
|---|---|---|---|
| 1, 5 | CTAB-based | 5.5 | 30 |
| 2, 6 | CTAB-based | 6.5 | 30 |
| 3, 7 | CTAB-based | 5.5 | 45 |
| 4, 8 | CTAB-based | 6.5 | 45 |
| 9–11 | TNS-based | 6.5 | 45 |

All samples were applied to agarose gel electrophoresis (Figure 3.2a). Bands with an approximate length of 1 kb were observed, which were likely 16S rRNAs. This analysis was not done with an denaturing gel, therefore the secondary structure of the 16S rRNA allows it to move faster through the gel and the bands appear to be smaller than their actual size (which is approximately 1.5 kb). Very light bands were also visible above the 16S rRNA bands and above the lowest band of the DNA ladder which were most likely 23S and 5S rRNAs, respectively. The smear at the top of each lane was genomic DNA (gDNA). With the TNS-based method most gDNA fragments had sizes above 4–5 kb or even outside of the DNA ladder's range (> 10 kb). With the CTAB-based method the gDNA was more fragmented; most fragments had a size between 3 and 8 kb. On examination of the extract's colouring (Figure 3.2b, c), a correlation with the beat beading parameters was observed. Longer bead beating times and higher speeds lead to stronger colouring, which is an indication of humic acids coprecipitation. The clearest extracts were gained with bead beating for 30 seconds at a speed setting of 5.5 m $s^{-1}$.

The combined DNA and RNA extracts were then simultaneously separated and purified with the All-Prep DNA/RNA Mini Kit. No DNA was visible in RNA extracts and vice versa after visualisation with agarose gel electrophoresis (data not shown). DNA and RNA aliquots were quantified with Invitrogen's Quant-iT™ assays (Figure 3.3). DNA yields were clearly affected by bead beating parameters, where increased time and speed were directly correlated to the amount of DNA extracted (CTAB-based method). A distinct difference in DNA yield could also be observed when comparing the different protocols. At the same settings (6.5 m $s^{-1}$, 45 s) the yields were higher with the CTAB-based method. RNA extraction efficiencies showed an indirect correlation to the bead beating intensity with the best yields at settings of 30 s long bead beating at a speed of 5.5 m $s^{-1}$ (CTAB-based method). The TNS-based approach resulted in more RNA amounts than the CTAB-based method at the same settings (6.5 m $s^{-1}$, 45 s), but with the most efficient bead beating parameters the CTAB-based method yielded about five times more RNA.

Testing for *Desulfosporosinus*-specific extraction biases was done by applying 5 ng of DNA extract to 16S qPCR assays targeting total *Bacteria* & *Archaea* and *Desulfosporosinus*. Only minimal differences in the 16S rRNA gene copy numbers in both assays were observed, well within the range of standard deviations of the technical replicates (Figure 3.4). The measured 16S rRNA gene copy numbers were $5.8 \times 10^5 \pm 4.2 \times 10^4$ copies (ng DNA)$^{-1}$ (7 % RSD[1]) and $3.1 \times 10^4 \pm 6.9 \times 10^3$ copies (ng DNA)$^{-1}$ (22 % RSD), respectively. The average ratio of *Desulfosporosinus* to total *Bacteria* & *Archaea* 16S rRNA copy numbers was 5.2 ± 1.0 % (19 % RSD). When comparing these RSDs to the RSDs of the technical replicates in the *Desulfosporosinus*-specific 16S rRNA qPCR assay (Figure 3.4b), it can be concluded that no distinct

[1]relative standard deviation

difference can be seen between the different protocols and parameters. It must be noted, that for the quantification of *Bacteria & Archaea*, the primer 1389Farch was used instead of 1389Fmix, which has a smaller target range. But, as described in Section 3.1.5.3, there apparently is no difference between those two primers when used with this qPCR assay.

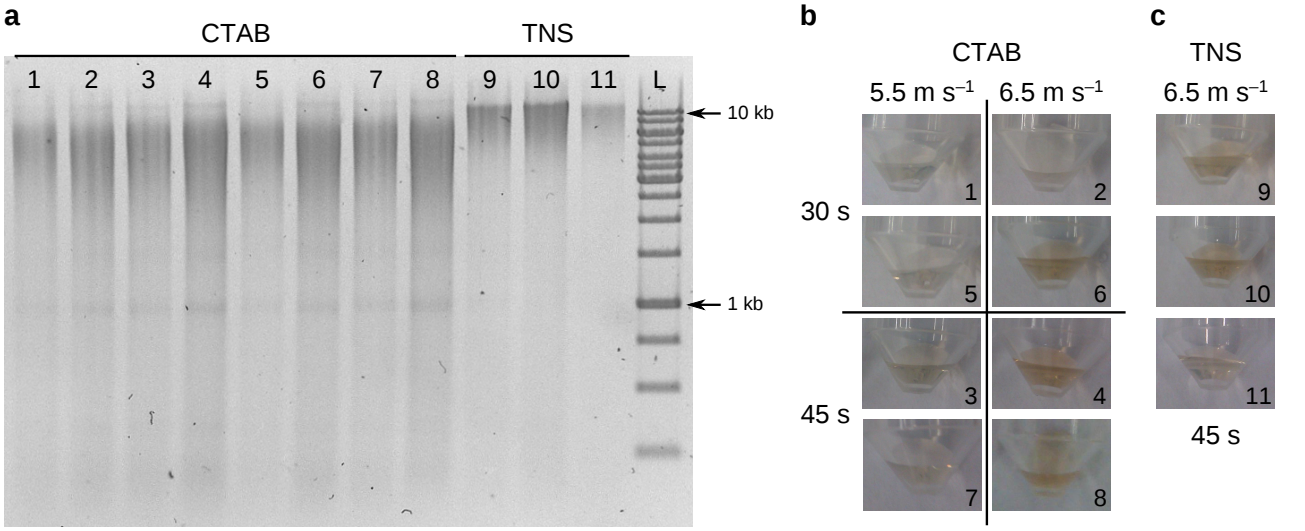


**Figure 3.2** Comparison of TNS- and CTAB-based extraction methods. 1–8 are CTAB-based extracts under different bead beating parameters. 9–11 are TNS-based extracts. **(a)** Agarose gel electrophoresis of the combined DNA and RNA extracts (1 % agarose, 120 V, 50 min; *L* GeneRuler™ 1 kb DNA Ladder, arrows indicate bands with sizes of 1 and 10 kb). **(b)** Photos of the CTAB-based extracts given as a table with bead beating parameters as column and row labels (speed and time, respectively). **(c)** Photos of the TNS-based extracts. Photos were taken of samples inside 2 mL reaction tubes against white paper.

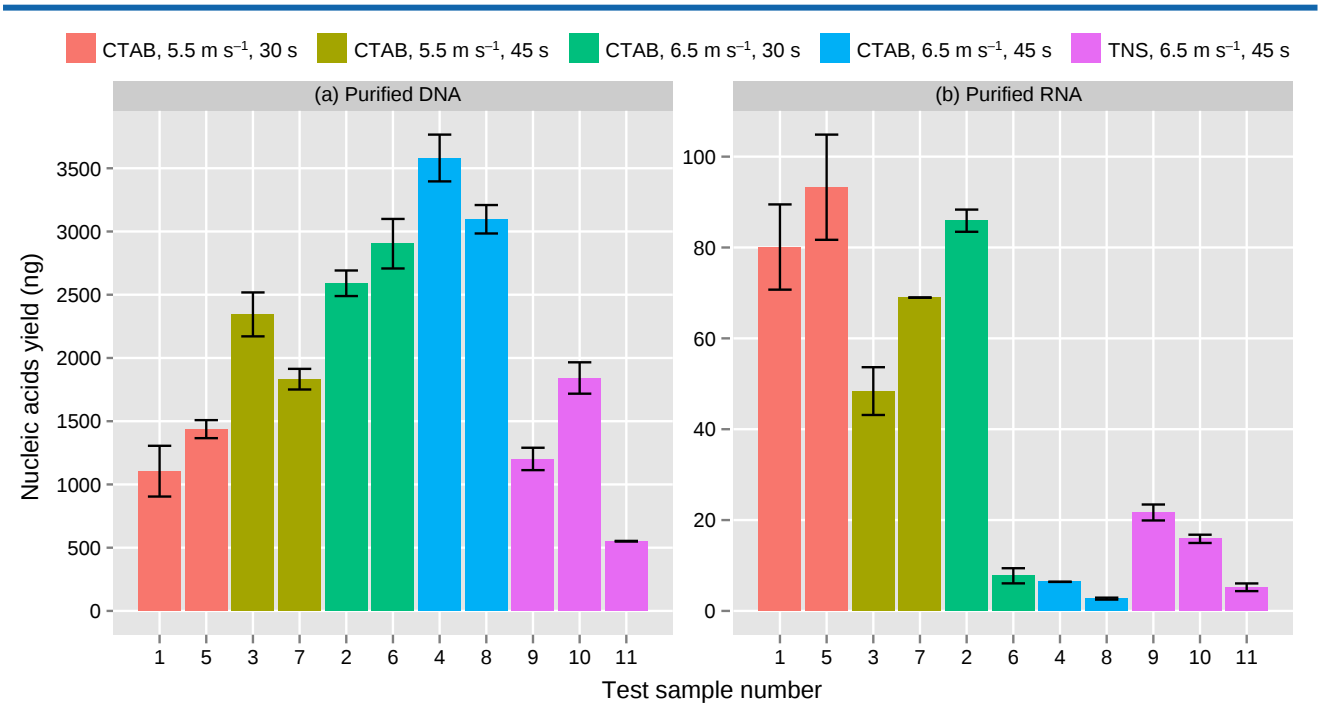


**Figure 3.3** Quantification of DNA (a) and RNA (b) samples done with the Quant-iT™ PicoGreen® dsDNA and RiboGreen® RNA kits. Standard curve quality indicators: $R^2$ = 1.000 and 0.993, respectively. Error bars are standard deviations of technical replicates in the Quant-iT™ assays (n = 2). Extraction methods and bead beating parameters are colour coded. *CTAB* CTAB-based extraction protocol; *TNS* TNS-based extraction protocol.

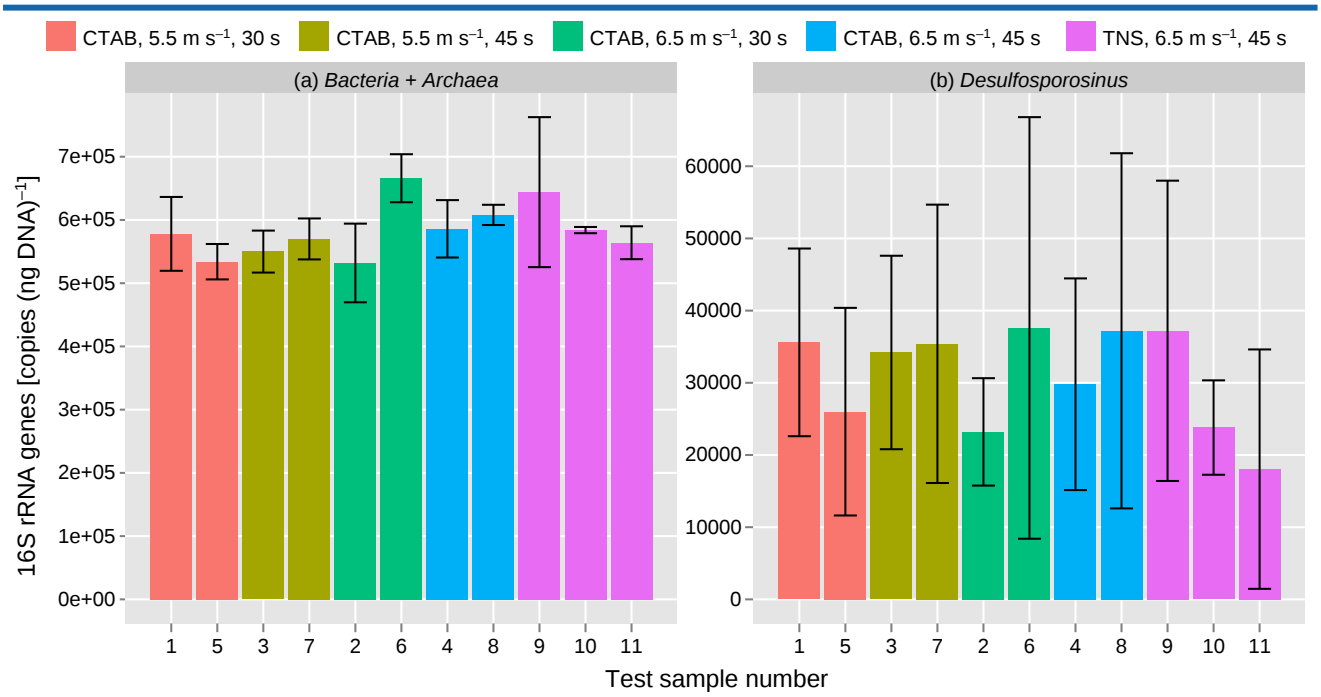


**Figure 3.4** Different nucleic acids extraction methods and parameters analysed with 16S rRNA qPCR assays targeting total *Bacteria* & *Archaea* (a) and *Desulfosporosinus* (b). Error bars are standard deviations of technical replicates in the qPCR assays (n = 3). Extraction methods and bead beating parameters are colour coded. *CTAB* CTAB-based extraction protocol; *TNS* TNS-based extraction protocol. **(a)** qPCR standard curve quality indicators: $R^2$ = 0.994, PCR efficiency = 99.6 %. Primer 1389Farch was used instead of 1389Fmix in this assay. **(b)** qPCR standard curve quality indicators: $R^2$ = 0.996, PCR efficiency = 69.1 %.

### 3.1.2. Nucleic acids extraction improvements

This section describes the steps which were taken to (1) improve RNA integrity and extraction yields and (2) improve the removal of PCR inhibitors.

#### 3.1.2.1. Grinding in liquid nitrogen and Lysing Matrix tubes

Microcosm 25 samples were ground in liquid nitrogen and transferred directly to Lysing Matrix A or E tubes. Lysing Matrix A tubes were used without the ceramic sphere. As a control, samples were extracted without prior grinding in liquid nitrogen in both types of bead beating tubes as well (Table 3.2). The CTAB-based protocol was the applied extraction method, followed by separation with the AllPrep DNA/RNA Mini Kit. In the total nucleic acids extracts, no difference in colouring was observed between the different treatments. 16S rRNA bands were visible and after separation no endogenous DNA contaminations were observed in RNA samples when analysed with agarose gel electrophoresis (data not shown).

**Table 3.2** Samples used for tests of liquid nitrogen grinding and different Lysing Matrix tubes. Given numbers are used in the following figures, but are not related to test sample numbers used in Section 3.1.1.

| Number | Grinding in liquid nitrogen | Type of Lysing Matrix tube |
|---|---|---|
| 1, 2 | no | A |
| 3, 4 | no | E |
| 5, 6 | yes | E[a] |
| 7, 8 | yes | E |
| 9, 10 | yes | A |

[a]Samples were flash frozen in Lysing Matrix A tube, then ground in liquid nitrogen and bead beat in a Lysing Matrix E tube.

Differences in RNA extraction yields were analysed by quantifying all RNA samples with Invitrogen's Quant-iT™ assay (Figure 3.5). With bead beating in Lysis Matrix E tubes, only minimal differences in RNA yields between samples ground in liquid nitrogen and directly extracted were observed. Grinding in liquid nitrogen, however, improved the RNA extraction efficiency in combination with Lysing Matrix A tubes. Comparison of yields between the both bead beating tube types clearly showed that Lysing Matrix E was more suitable for the Schlöppnerbrunnen soil slurry samples.

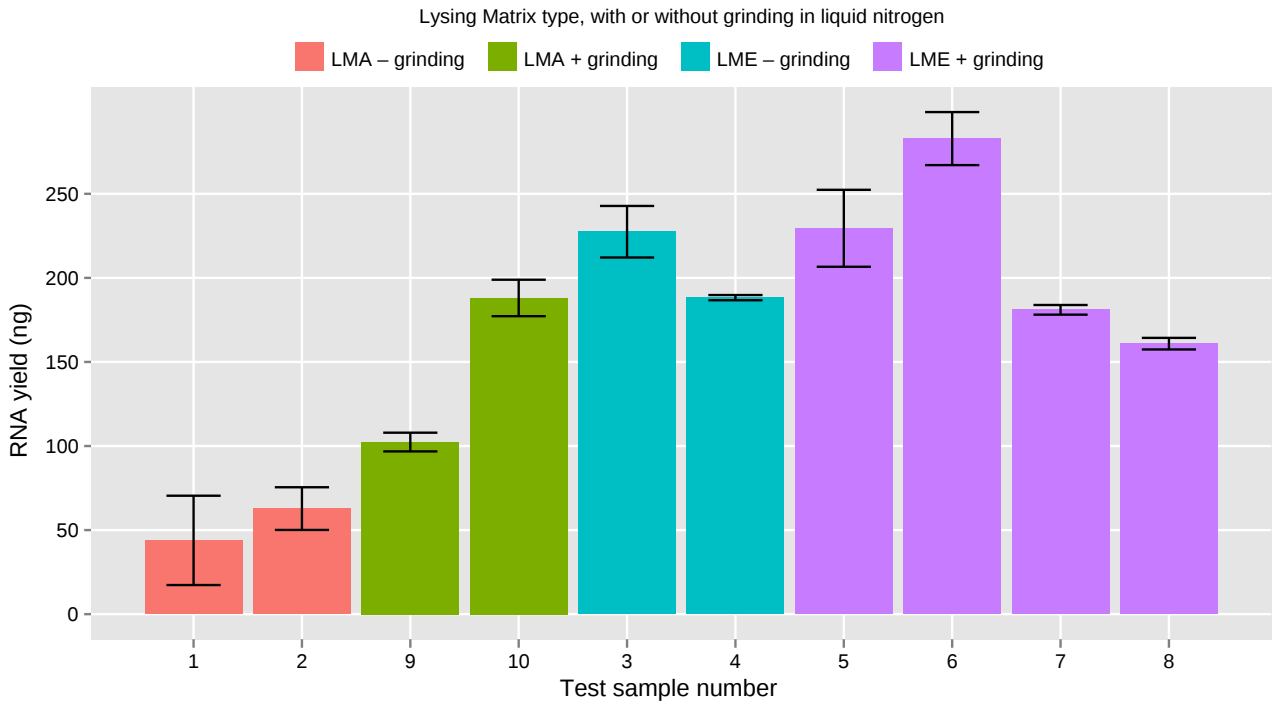


**Figure 3.5** Quantification of RNA samples done with the Quant-iT™ RiboGreen® RNA kit. Standard curve quality indicators: $R^2 = 0.994$. Error bars are standard deviations of technical replicates in the Quant-iT™ assay (n = 3). *LMA* Lysing Matrix A; *LME* Lysing Matrix E.

### 3.1.2.2. OneStep™ PCR Inhibitor Removal Kit

Test samples were ground in liquid nitrogen, extracted with the CTAB-based protocol and then half of them were additionally applied to the OneStep™ PCR Inhibitor Removal Kit. Test samples were chosen from different time points (time point zero and very long incubated) and of different origins (untreated versus soil slurry) to test if this would have an effect in the extraction process (Table 3.3). DNA and RNA aliquots were directly digested with RNase and DNase, respectively. In all steps where samples were pelleted, a trend, that the extract colouring was stronger in the microcosm 25 samples, was observed.

**Table 3.3** Test samples from the incubation time point zero and microcosm 25 were extracted and half the samples were purified with the OneStep™ PCR Inhibitor Removal Kit. Given numbers are used in the following figures, but are not related to test sample numbers used in previous sections.

| Number | Type of test sample | Purified with OneStep™ PCR Inhibitor Removal Kit |
|---|---|---|
| 1, 2 | Time point zero | no |
| 3, 4 | Time point zero | yes |
| 5, 6 | Microcosm 25 | no |
| 7, 8 | Microcosm 25 | yes |

All samples were quantified with Invitrogen's Quant-iT™ assays to test for nucleic acids loss from using the OneStep™ PCR Inhibitor Removal Kit. On average the DNA yield was lower in purified samples, but the RNA yield higher (Figure 3.6). The average decrease in DNA yield was 27 % and 34 % for time point zero and microcosm 25 test samples, respectively, which can be explained through loss of DNA in the purification step. What can not be easily explained was the average increase in RNA yield of 67 % and 92 % for time point zero and microcosm 25 test samples, respectively.

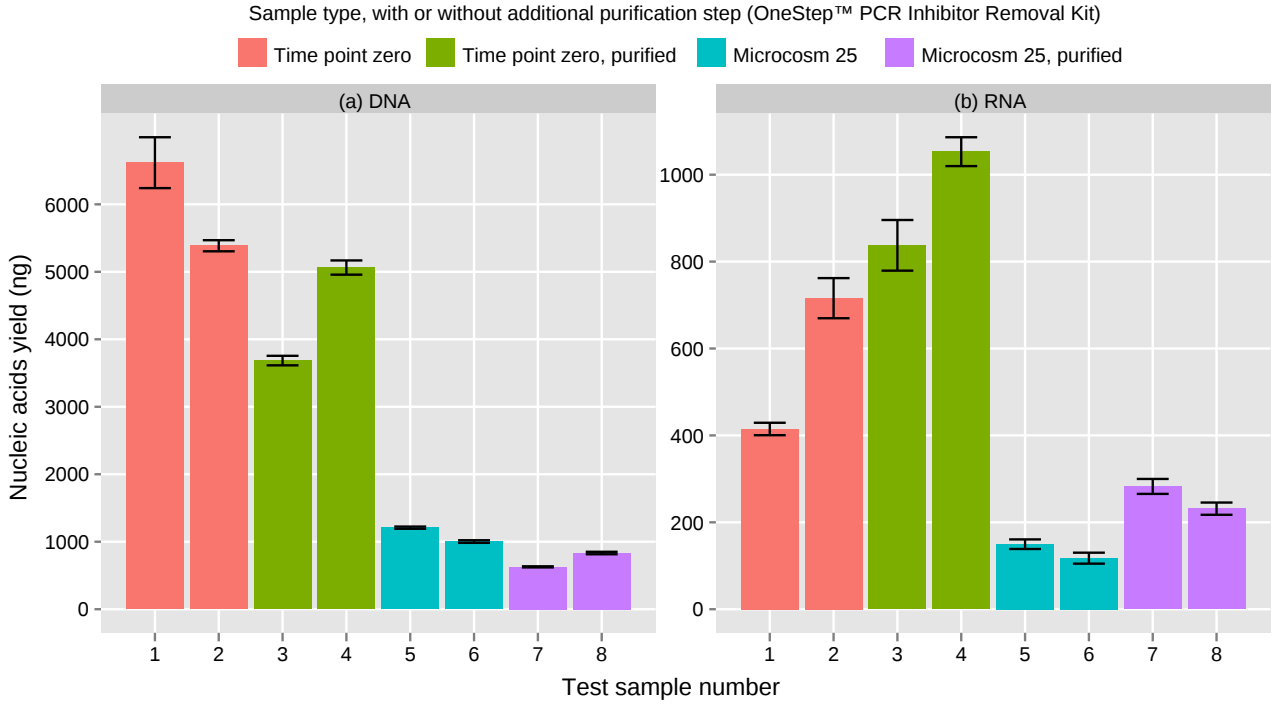


**Figure 3.6** Quantification of DNA (a) and RNA (b) samples done with the Quant-iT™ PicoGreen® dsDNA and RiboGreen® RNA kits. Standard curve quality indicators: $R^2$ = 0.999 and 0.993, respectively. Error bars are standard deviations of technical replicates in the Quant-iT™ assays (n = 3).

The NTC in 16S rRNA qPCR assays targeting total *Bacteria* & *Archaea* always gave an amplification signal at late quantification cycles. Since broad-coverage primers were used, trace amounts of any bacterial or archaeal DNA in either plasticware or buffers could explain that. It can therefore be said that all samples free of inhibitory substances have a quantification cycle ($C_q$) smaller than or equal to the NTC's $C_q$, and samples which result in $C_q$s higher than that of the NTC are inhibited to a certain degree. This phenomenon was used to test RNA samples for PCR inhibitors. Figure 3.7 shows the analysis of time point zero samples, where two samples where additionally purified with the OneStep™ PCR Inhibitor Removal Kit (Table 3.3). Total inhibition was observed in the not-purified samples (blue traces), when 2 µL of sample were applied to the qPCR, however, 2 µL of 1:10 dilutions of the same samples removed the inhibitory effect. The purified samples (undiluted and diluted, green traces) were

similarly amplified at the same $C_q$ as the NTC, indicating that they are free of PCR inhibitors and endogenous DNA. It is possible, but unlikely, that a combination of DNA contamination and PCR inhibition would return the same results, since a DNA contamination causes lower $C_q$s and PCR inhibition causes higher $C_q$s. Melt curve analysis of this assay showed peaks at different temperatures, but no pattern could be determined (Figure 3.7c, d). The variability in melt peaks is most likely due to the fact that undefined contaminations are amplified. The same qPCR test was done for microcosm 25 test samples and no difference between purified and untreated samples could be observed. Total inhibition was observed when undiluted samples were applied to the qPCR assay, but the result of diluted samples was the same as with the time point zero samples ($C_q$ like the NTC).

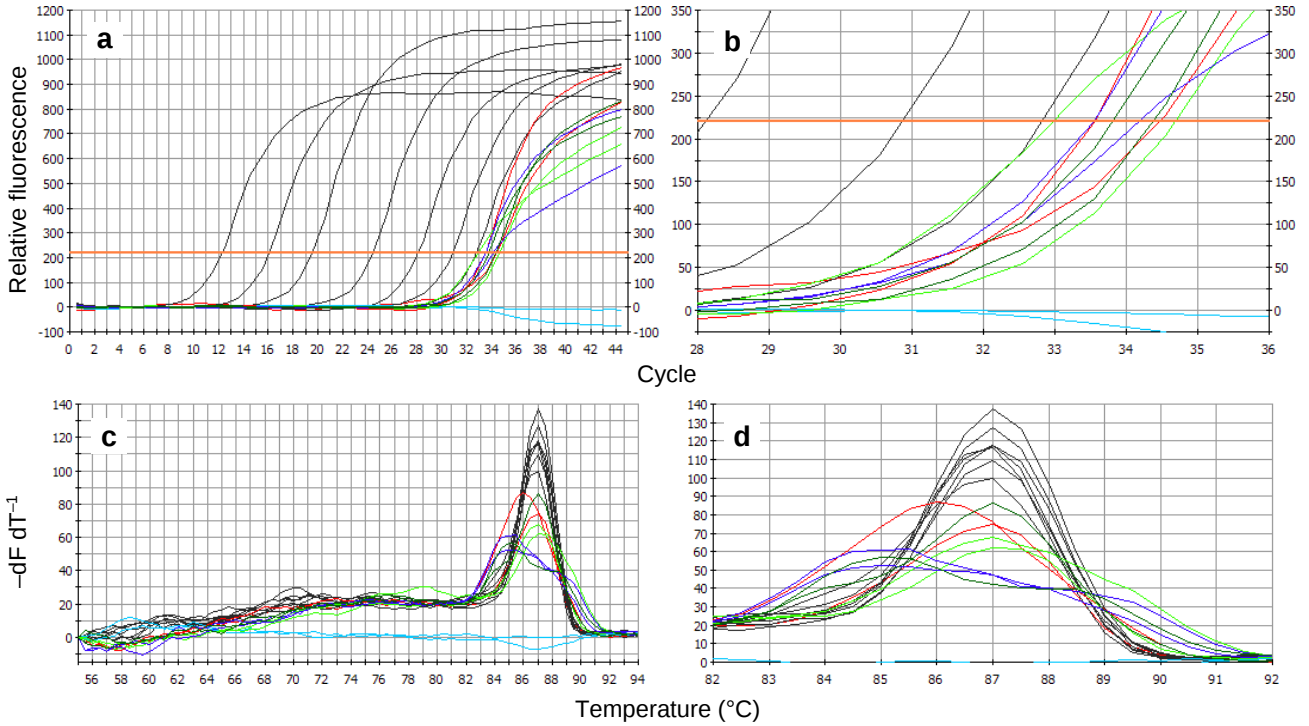


**Figure 3.7** **(a)** 16S rRNA qPCR assay targeting total *Bacteria* & *Archaea* with RNA test samples 1–4. Traces of standard dilution series (black) and NTCs (red) are shown. Time point zero samples 1 and 2 (without additional purification step) are displayed in blue and 3 and 4 (with additional purification step) in green. 2 or 0.2 µL of RNA samples were used as templates (light and darker colouring, respectively). The horizontal, orange line indicates the threshold used for determination of quantification cycles. qPCR standard curve quality indicators: $R^2$ = 0.994, PCR efficiency = 90.9 %. Primer 1389Farch was used instead of 1389Fmix in this assay. **(b)** Magnified section from (a). **(c)** Melt curve analysis of the same samples. −dF $dT^{-1}$ is the negative first derivative of fluorescence versus temperature. **(d)** Magnified section from (c).

### 3.1.3. DNA and RNA separation methods

Complete separation of DNA from RNA was essential, since traces of DNA can not be distinguished from reverse transcribed RNA (cDNA) when analysed with (q)PCR.

#### 3.1.3.1. AllPrep DNA/RNA Mini Kit, Lithium chloride precipitation, and nuclease treatments

After sufficient microcosm 25 test samples were extracted with the CTAB-based protocol (Section 3.1.1; for this evaluation the additional extraction improvements mentioned in Section 3.1.2 were not used), the following separation methods were compared: (1) AllPrep DNA/RNA Mini Kit with and without DNase digestion, (2) Lithium chloride (LiCl) precipitation with and without DNase digestion and (3) direct DNAse digestion (Table 3.4). Samples were analysed, before and after separation, with agarose gel electrophoresis. No difference between total nucleic acids extracts and DNA aliquots was observed.

rRNA bands could not be detected in any of the samples. No genomic DNA was visible in RNA aliquot lanes (data not shown).

**Table 3.4** List of all tested separation methods. Given numbers are used in the following figures, but are not related to test sample numbers used in previous sections.

| Number | Separation method |
|---|---|
| 1, 2 | AllPrep DNA/RNA Mini Kit |
| 3, 4 | AllPrep DNA/RNA Mini Kit with on-column DNase treatment |
| 5, 6 | Lithium chloride precipitation |
| 7, 8 | Lithium chloride precipitation, followed by DNase treatment |
| 9, 10 | DNase digestion of half of the nucleic acids extract |
| — | TRIzol® reagent, separate test samples, see Section 3.1.3.3 |

Exact quantification was done only for RNA samples (Figure 3.8). The highest yields were achieved with the AllPrep DNA/RNA Mini Kit and the direct DNase digestion protocol. However, the combination of the AllPrep DNA/RNA Mini Kit with a DNase treatment step removed nearly all RNA from the sample. Lithium chloride precipitation without DNase digestion step yielded less RNA than the AllPrep DNA/RNA Mini Kit or direct DNase digestion protocol. When lithium chloride precipitation and DNase treatment where combined, the results were inconsistent. One replicate's RNA yield was in the same range as the RNA yield from samples that were not treated with DNase, while no RNA could be recovered in the other replicate.

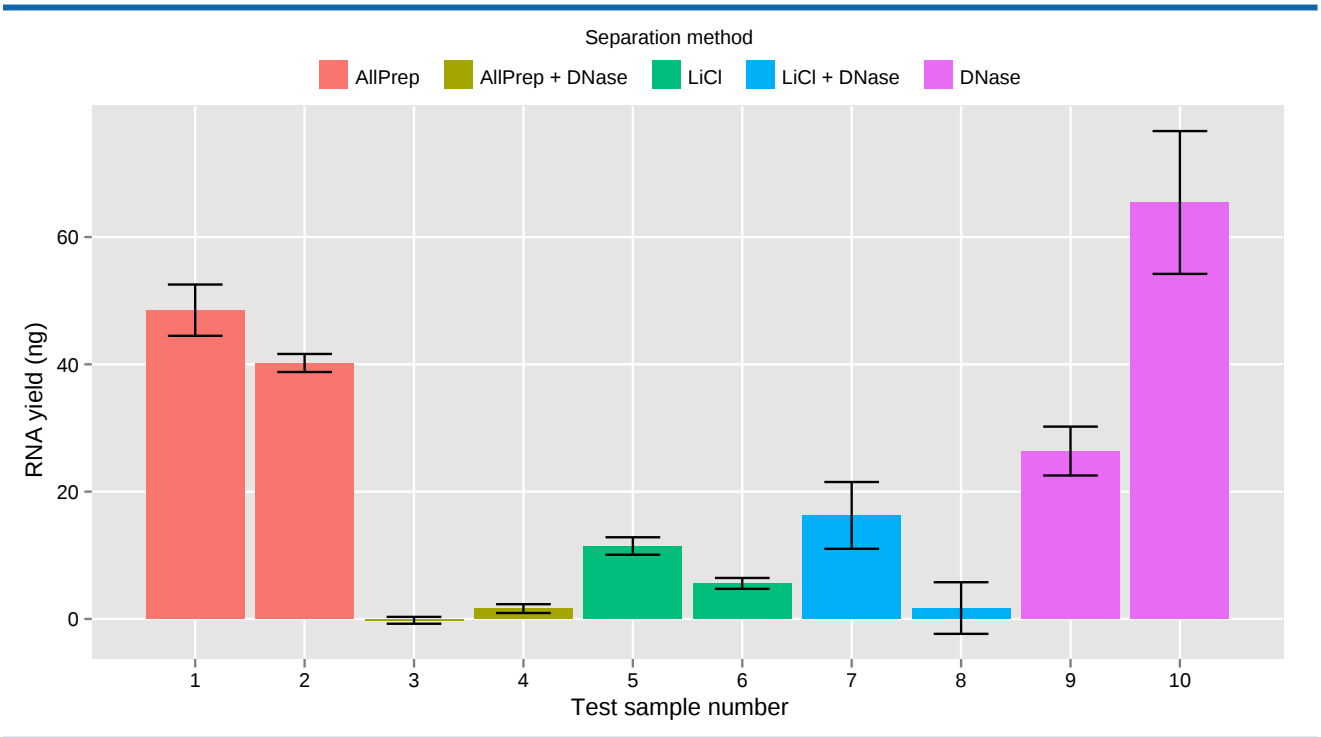


**Figure 3.8** Quantification of RNA samples done with the Quant-iT™ RiboGreen® RNA kit. Standard curve quality indicators: $R^2$ = 0.997. Error bars are standard deviations of technical replicates in the Quant-iT™ assay (n = 3). *AllPrep* AllPrep DNA/RNA Mini Kit; *LiCl* Lithium chloride precipitation; *DNase* DNase treatment.

RNA samples were further controlled for DNA contamination and PCR inhibitory substances. All samples were applied to the control PCR described in Section 2.11.2. In addition, the same RNA samples were applied to the PCR mixed with 1 ng of positive control DNA. DNA contamination would present as a band at approximately 123 bp in the RNA-only sample. Inhibition of PCR would present as *no*

band in the RNA + positive control DNA lanes. Slight bands from DNA contaminations were observed in samples separated with lithium chloride precipitation without DNase digestion step (Figure 3.9). This was to be expected, since the RNA was precipitated and washed in the original combined DNA and RNA extract tube, while the supernatant, which contained the DNA, was transferred to a new tube. The supernatant can of course not be transferred completely, since traces of liquid remain on the wall and in the pellet.

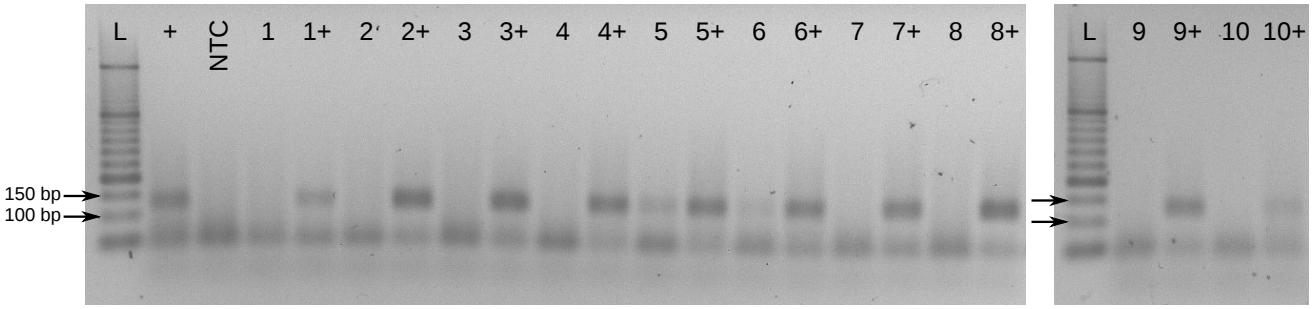


**Figure 3.9** Agarose gel electrophoresis of the PCR for endogenous DNA contamination testing (2.5 % agarose, 120 V, 50 min). *L* O'RangeRuler 50 bp DNA Ladder, arrows are 100 and 150 bp; *+* positive control, 1 ng of genomic DNA from soil; *NTC* NTC; *1–10* undiluted RNA sample; *1+–10+* inhibition controls, undiluted RNA sample + 1 ng of genomic DNA from soil. Target bands have approximately 123 bp. Bands visible at the bottom of the gel are most likely primer dimers.

### 3.1.3.2. DNA contaminations in the AllPrep DNA/RNA Mini Kit

During further evaluation steps the RNA from samples extracted and separated with the CTAB-based protocol and the AllPrep DNA/RNA Mini Kit was also tested with qPCR for DNA contamination. No positive signal was expected, because this analyses was already done with standard PCR (Figure 3.9). However, this was not the case. The step with the AllPrep DNA/RNA Mini Kit was identified as the source of this DNA contamination. Figure 3.10 shows the qPCR analysis of nucleic acids-free samples, which were applied to the AllPrep DNA/RNA Mini Kit. The $C_q$ value is lower in the "RNA" than in the "DNA" aliquot, and a clear difference of both sample types to the NTC was observed.

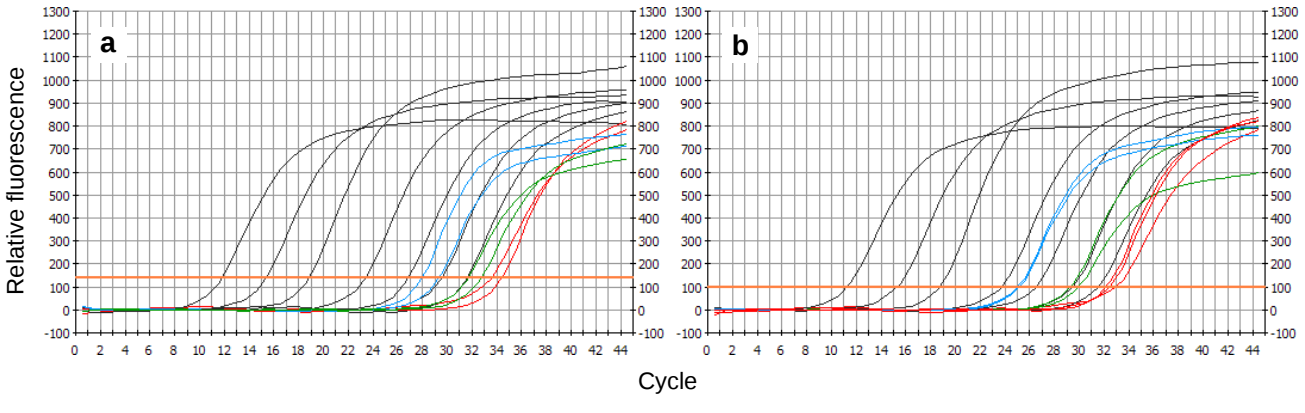


**Figure 3.10** **(a)** Bacterial or archaeal DNA contamination introduced in the separation step with the AllPrep DNA/RNA Mini Kit. 30 µL of DEPC-treated water were used as a "sample" (two replicates). The resulting eluates were analysed with a 16S rRNA qPCR assay targeting total *Bacteria & Archaea*. Traces of standard dilution series (black), NTCs (red) and "DNA" and "RNA" eluates (green and blue, respectively) are shown. The horizontal, orange line indicates the threshold used for determination of quantification cycles. qPCR standard curve quality indicators: $R^2$ = 0.995, PCR efficiency = 95.8 %. Primer 1389Farch was used instead of 1389Fmix in this assay. **(b)** The analysis was repeated using a new kit with a different lot number (with 50 µL of DEPC-treated water as a "sample"). qPCR standard curve quality indicators: $R^2$ = 0.994, PCR efficiency = 93.9 %.

### 3.1.3.3. TRIzol® reagent

The TRIzol® reagent protocol, with and without adding glycogen at the RNA precipitation step, was tested for separation of nucleic acids. Microcosm 25 soil samples, extracted with both the TNS- and CTAB-based methods (CTAB-based protocol was done at two different bead beating speeds: 5.0 and 6.5 m $s^{-1}$), were used and the resulting aliquots were analysed with agarose gel electrophoresis. The DNA was completely lost in all samples. Weak RNA bands were visible in the CTAB-based extracts and even weaker in the TNS-based sample (Figure 3.11).

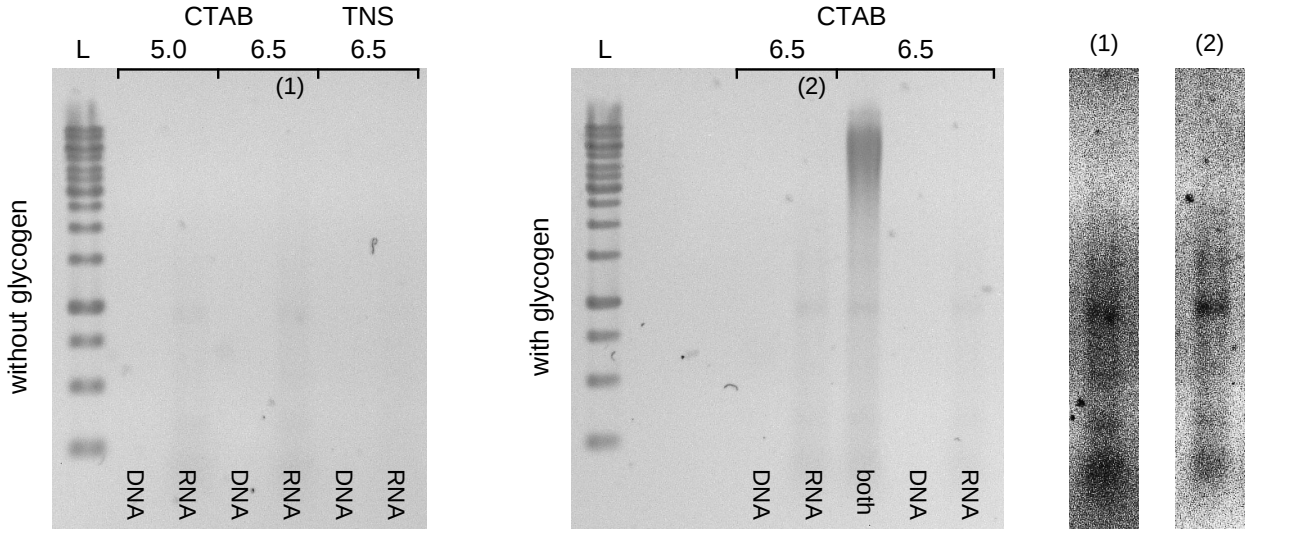


**Figure 3.11** Nucleic acids test samples extracted with CTAB- or TNS-based protocols (values given indicate the bead beating speed in m $s^{-1}$; bead beating time was 45 s) and separated with the TRIzol® reagent with or without glycogen addition (1 % agarose, 120 V, 50 min; *L* GeneRuler™ 1 kb DNA Ladder; *both* total nucleic acids extract; *DNA* DNA aliquot; *RNA* RNA aliquot). Two lanes (1, 2) are shown with very high contrast settings to highlight the presence of weak 16S rRNA bands.

## 3.1.4. Reverse transcription of RNA

Two kits by Invitrogen based on their propitiatory SuperScript® III Reverse Transcriptase were used. The SuperScript® VILO™ cDNA Synthesis Kit was briefly tested, but then abandoned in favour of the SuperScript™ III First-Strand Synthesis System for RT-PCR kit. All results presented here were done with this kit.

### 3.1.4.1. RNA dilution series

Even after purification steps, samples can and will contain inhibitors, either from endogenous or exogenous sources. Inhibitors can interfere with the reverse transcription, the (q)PCR reaction, or both. Two RNA samples were tested for inhibition during this two steps, one sample was from time point zero and one from microcosm 25. They were extracted with the CTAB-based protocol and the OneStep™ PCR Inhibitor Removal Kit, followed by direct nuclease treatments. Different amounts of RNA were then reverse transcribed and constant amounts of cDNA applied to the qPCR. The results of the dilution series from the time point zero test sample are shown in Figure 3.12. In this sample, inhibition was only observed when 5 µL of undiluted sample (= 52.5 ng of RNA, filled dots in Figure 3.12b, d) were analysed, but not at smaller volumes (≤ 1 µL). The average total *Bacteria* & *Archaea* and *Desulfosporosinus* 16S rRNA copy numbers were $4.2 \times 10^6 \pm 1.4 \times 10^6$ copies (ng RNA)$^{-1}$ (33 % RSD) and $2.8 \times 10^5 \pm 6.9 \times 10^4$ copies (ng RNA)$^{-1}$ (24 % RSD), respectively, which corresponds to a *Desulfosporosi-*

*nus* to *Bacteria* & *Archaea* 16S rRNA transcripts ratio of 6.8 % (calculated without outliers). This is quite a high ratio, considering that the 16S rRNA *gene* ratio was only 0.010 % at time point zero (Figure 3.19).

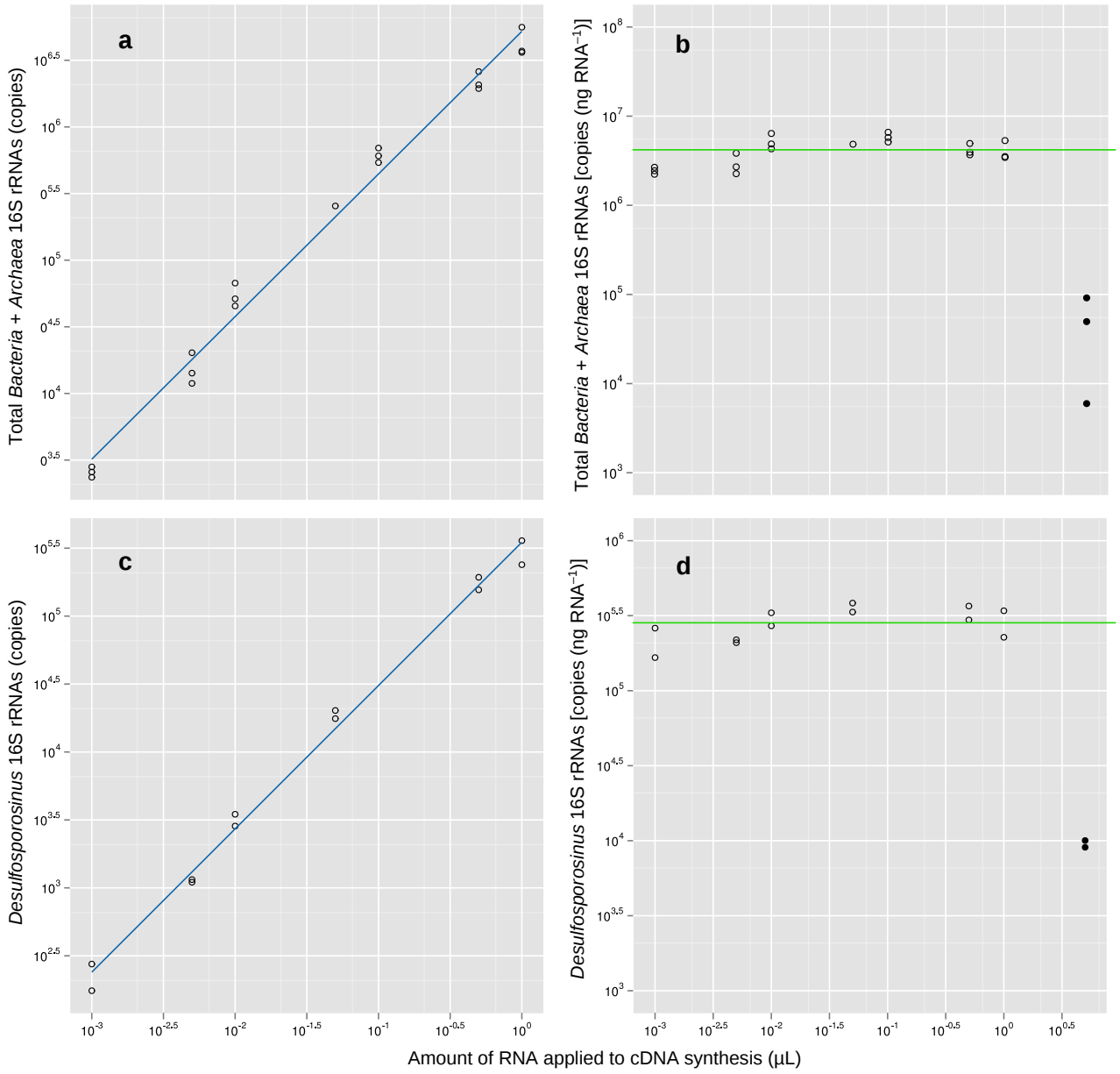


**Figure 3.12** Different amounts of purified and DNA-free RNA from time point zero were reverse transcribed (concentration = 10.5 ng $\mu L^{-1}$). 2 out of 20 µL cDNA solution were then analysed with the 16S rRNA qPCR assays. The data is plotted in absolute copy numbers (plots on the left) and in copy numbers relative to 1 ng of reverse transcribed RNA (plots on the right). The qPCR results of the largest applied volume (5 µL of RNA aliquot) are also shown as filled circles in the right plots to visualize that they are clearly outliers. Linear regression analysis of 16S rRNA copy numbers against reverse transcribed volume of RNA extract are shown as blue lines. The horizontal green lines represent the average 16S rRNA gene copy number of this sample (calculated without outliers). **(a)** and **(b)** 16S rRNA qPCR assay targeting total *Bacteria* & *Archaea*. qPCR standard curve quality indicators: $R^2$ = 0.993, PCR efficiency = 92.3 %. Dilution series linear regression quality indicator: $R^2$ = 0.942. Primer 1389Farch was used instead of 1389Fmix in this assay. **(c)** and **(d)** 16S rRNA qPCR assay of the same dilution series targeting *Desulfosporosinus*. qPCR standard curve quality indicators: $R^2$ = 0.999, PCR efficiency = 85.8 %. Dilution series linear regression quality indicator: $R^2$ = 0.947.

The average total *Bacteria* & *Archaea* and *Desulfosporosinus* 16S rRNA copy numbers of the microcosm 25 test sample were $1.6 \times 10^6 \pm 6.8 \times 10^5$ copies $(\text{ng RNA})^{-1}$ (43 % RSD) and $2.3 \times 10^7 \pm 5.1 \times 10^6$ copies $(\text{ng RNA})^{-1}$ (22 % RSD), respectively, which corresponds to a *Desulfosporosinus* to *Bacteria* & *Archaea* 16S rRNA transcripts ratio of 1465 % (calculated without outliers, Figure B.1). In theory, a ratio above 100 % is impossible, meaning that either the *Bacteria* & *Archaea* or the *Desulfosporosinus*-specific assay introduced bias into this analysis. As with the time point zero sample, inhibition was only seen when 5 µL of undiluted RNA (= 14 ng of RNA, filled dots in Figure B.1d) were analysed. Outliers were also observed with lower RNA amounts in the 16S rRNA qPCR assay targeting *Bacteria* & *Archaea*, indicating that this assay is unstable at this low concentrations. Based on this data, it was decided to use 1 ng of RNA (concentration of the extract must be ≥ 1 ng $\mu L^{-1}$) for analysis with RT-qPCR to exclude inhibitory effects.

#### 3.1.4.2. Reproducibility

To evaluate the tube-to-tube differences in the cDNA synthesis, the same time point zero sample as used in the RNA dilution series was reverse transcribed in triplicates, followed by quantification. With the 16S rRNA qPCR assay targeting total *Bacteria* & *Archaea* a relative standard deviation between the triplicate's copy numbers of 21 % was calculated. The RSD of the technical qPCR replicates was between 12–58 %. The same analyses was done with *Desulfosporosinus*-specific 16S rRNA qPCR assay, resulting in a RSD of 5 % (11–17 % for technical qPCR replicates). For both qPCR assays, the RSD of the reverse transcribed replicates was within or smaller than the range of the RSD from technical qPCR replicates, therefore the reverse transcription was deemed reproducible.

### 3.1.5. qPCR evaluation

#### 3.1.5.1. DNA dilution series

Depending on the sample, the extraction procedure yielded different amounts of nucleic acids. Every extract was diluted to the same concentration for normalisation purposes, which also changed the amount of possible inhibitory substances. To verify the absence of qPCR inhibitors in DNA aliquots (at relevant dilution factors), a soil slurry sample from the long-term incubations (number 25, day 9) was analysed in an dilutions series (Figure 3.13). This extract was chosen because it was the incubation sample with the highest concentration and not the first or last sample in the time series (see Section 3.2.3). From all dilutions an average total *Bacteria* & *Archaea* 16S rRNA gene copy number could be calculated, which was $2.1 \times 10^6 \pm 4.5 \times 10^5$ copies $(\text{ng DNA})^{-1}$ (21 % RSD) and no inhibition could be detected within the tested dilution range (0.0049–5.0 µL, which corresponds to 0.059–61 ng). This analysis could not have been done with the *Desulfosporosinus*-specific 16S rRNA qPCR, since *Desulfosporosinus* was not abundant enough to be detected in diluted samples. For the final protocol, 5 ng of DNA per qPCR reaction was selected, since no inhibition was observed at that range, and 5 ng was also the amount used in a previous study (Pester *et al.*, 2010).

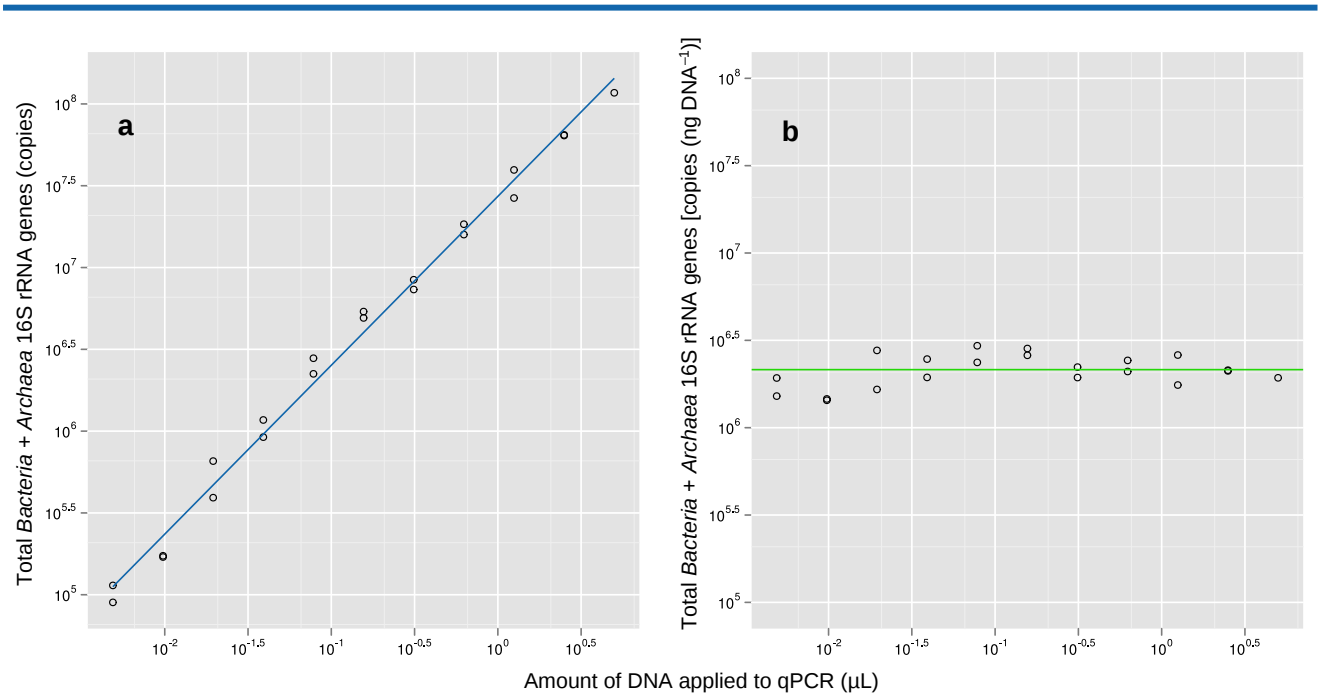


**Figure 3.13** Different amounts of purified DNA from soil slurry 25 (of day 9, concentration = 12.1 ng µL$^{-1}$) were quantified with 16S rRNA qPCR targeting total *Bacteria* & *Archaea*. The data is plotted in absolute gene copy numbers (a) and in gene copy numbers relative to 1 ng of DNA (b). Linear regression analysis of 16S rRNA gene copy numbers against volume of DNA extract is shown as a blue line. The horizontal green line represents the average 16S rRNA gene copy number of this sample. 0.0049–5.0 µL of DNA extract correspond to 0.059–61 ng. qPCR standard curve quality indicators: $R^2$ = 0.988, PCR efficiency = 89.5 %. Dilution series linear regression quality indicator: $R^2$ = 0.992.

### 3.1.5.2. cDNA dilution series

Manuals of reverse transcription kits recommended to dilute the finished cDNA solution at different degrees. For example, the manual of Invitrogen's SuperScript™ III First-Strand Synthesis System for RT-PCR kit recommends to use 2 µL cDNA in a 50 µL PCR reaction (4 %), and the manual of the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) states that the cDNA solution should not be more than 10 % of the total PCR reaction volume. Therefore, to test for effects of the reverse transcription components on the qPCR assay, an inhibition dilution series of one sample was analysed (the same sample was used in the DNA dilution series, slurry 25, day 9, Figure 3.14). Correlation between *Desulfosporosinus* 16S rRNA copy numbers and amount of cDNA applied to qPCR could be observed, with a normalised copy number of $9.0 \times 10^5 \pm 4.0 \times 10^5$ copies (ng RNA)$^{-1}$ (45 % RSD). No inhibitory effects were seen (highest tested volume was 1.25 µL in a 50 µL PCR reaction mix, 2.5 %). However, no correlation could be seen when the same sample was analysed with the qPCR assay targeting total *Bacteria* & *Archaea*. For the final RT-qPCR protocol, 0.5 µL (5 µL of a tenfold dilution) were used, which is a compromise between sensitivity (the bigger the applied cDNA solution volume, the better the sensitivity) and the possibility to perform multiple qPCR analysis (less applied cDNA solution increases number of possible qPCR assays that can be performed with one reverse transcribed sample).

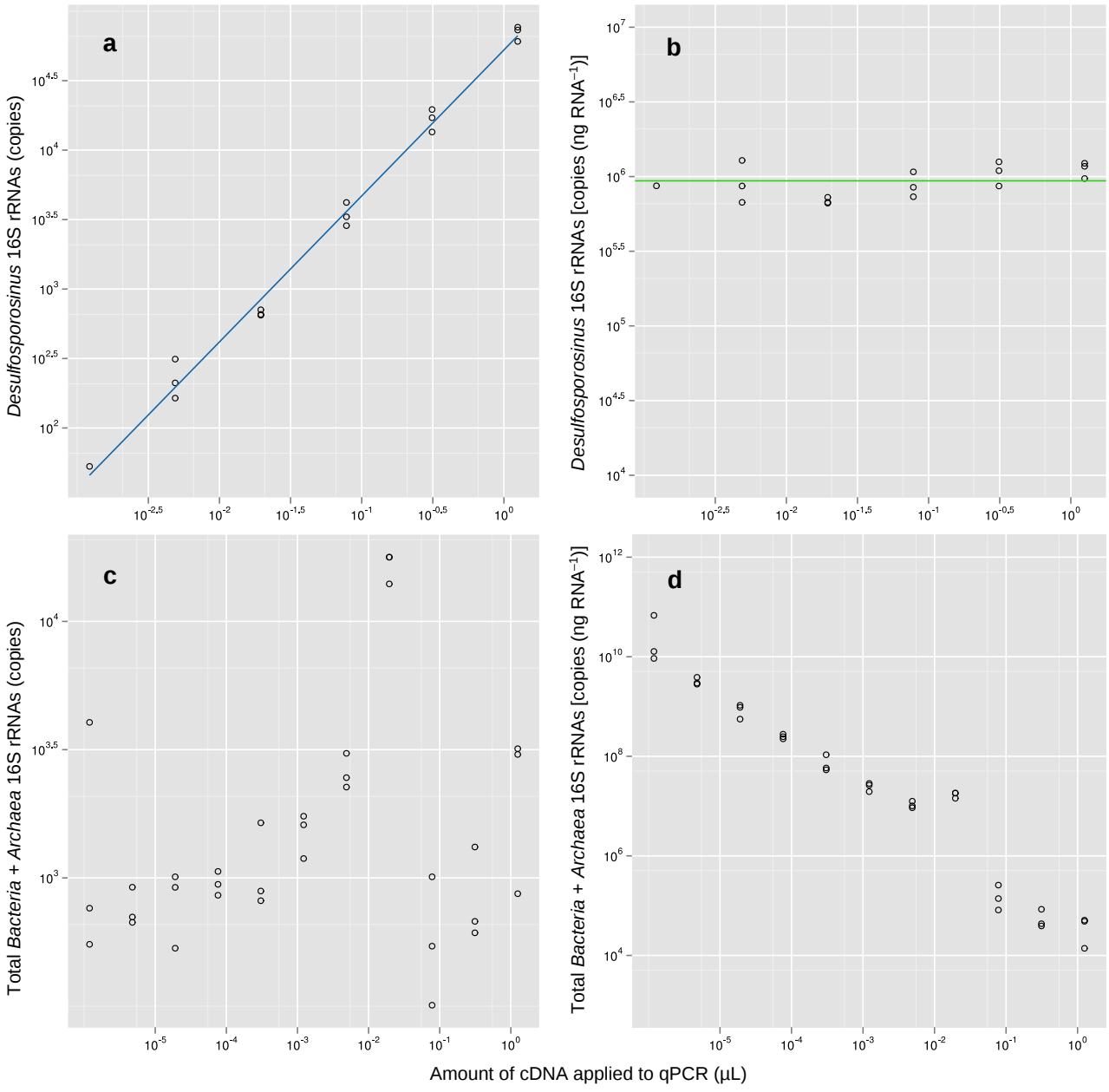


**Figure 3.14** 1 ng of purified and DNA-free RNA from soil slurry 25 (of day 9) was reverse transcribed. Different amounts of cDNA reaction solution were then analysed with the 16S rRNA qPCR assays. The data is plotted in absolute copy numbers (plots on the left) and in copy numbers relative to 1 ng of reverse transcribed RNA (plots on the right). **(a)** and **(b)** 16S rRNA qPCR assay targeting *Desulfosporosinus*. Linear regression analysis of 16S rRNA copy numbers against reverse transcribed volume of RNA extract is shown as a blue line. The horizontal green lines represents the average 16S rRNA copy numbers of this sample. qPCR standard curve quality indicators: $R^2$ = 0.997, PCR efficiency = 89.0 %. Dilution series linear regression quality indicator: $R^2$ = 0.985. **(c)** and **(d)** 16S rRNA qPCR assay of the same dilution series targeting total *Bacteria* & *Archaea*. Linear regression analysis and average 16S rRNA copy numbers are not plotted, since there is no correlation between cDNA amount and copy numbers. qPCR standard curve quality indicators: $R^2$ = 0.991, PCR efficiency = 89.5 %.

### 3.1.5.3. Comparison of the primers 1389Fmix and 1389Farch

The broad-coverage primers 1389F and 1389Farch differ in one base pair at the 5' end (third base, T vs C). Since some evaluation steps were done with the 1389Farch primer, it was compared to the degenerate primer 1389Fmix by analysing a *Bacteria* & *Archaea*-standard (*S. wolinii* 16S rRNA gene clone) dilution series ($10^{-3}$ to $10^{-8}$ dilutions) with both primers applied in parallel (the reverse primer was 1492R). The $C_q$ values of the highest and lowest standard dilutions were higher with the 1389Farch

primer than with the 1389Fmix primer by 2.7 %. $C_q$ values of the other dilutions were higher by 0.6–0.7 %, expect for the $10^{-3}$ dilution, where the $C_q$ value was lower with the 1389Farch primer by 0.6 %. In comparison, the average RSD of the dilution series' technical replicates with the 1389Fmix and 1389Farch primer were 1.3 % (0.3–2.0 %) and 0.9 % (0.0–2.2 %), respectively (n = 2). Therefore it can be said, that no meaningful difference in PCR amplification efficiency was observed between the two primers, even through the 1389F(mix) primer, in contrast to the 1389Farch primer, perfectly matches the standard DNA (see Section C.1).

## 3.2. Long-term peat soil incubations

During the incubations, more than 400 soil slurry samples were taken. Nucleic acids extraction and analysis with qPCR is not feasible within a reasonable time frame and budget with so many samples, therefore the determination of sulfate and substrate concentrations beforehand was necessary as a screening method. This was done with capillary electrophoresis and based on the results, microcosms and time points were selected for further analysis. Besides the screening purpose, the gained information also gave insights into the responses of the anaerobic microbial community in Schlöppnerbrunnen soil to sulfate and/or substrate supplementation. Sequential numbers were assigned to all soil slurries (see Table B.1) and are given in parentheses throughout this section for the sake of completeness.

### 3.2.1. Anoxic incubations

After the first weeks of incubation the distinctive smell of hydrogen sulfide was detectable while opening some bottles, which were incubated with sulfate and substrates, during soil sampling. The intensity of the hydrogen sulfide smell further increased over the following weeks.

The pH at the starting point of the incubation was 4.07. The average pH after eight weeks of incubation was 4.63 ± 0.23 (5.0 % RSD, minimum: 4.22, maximum: 4.98). Biological replicates amended with lactate and sulfate (numbers 14 and 15), and propionate and sulfate (numbers 20 and 21) showed average pH differences of 0.33 and 0.42, respectively. The control microcosms with sulfate (numbers 2 + 3) showed a difference of 0.19 and all other scenarios had pH values with less then 0.1 difference between their biological replicates (Table B.1).

### 3.2.2. Sulfate and substrate turnover

Sulfate and substrates were added regularly and their concentrations were measured after these additions. The resulting data can be interpreted in the following ways:

*Sulfate or substrate accumulation*
: No turnover of sulfate/substrates would results in their accumulation in the slurries, which can easily be detected with capillary electrophoresis. Over the course of the experiment a total of 1344 µmol $L^{-1}$ of sulfate was added to each sulfate-amended microcosm (928 µmol $L^{-1}$ until day 36). The total amount that was added for each substrate was 1320 and 1800 µmol $L^{-1}$ (2160 and 3120 µmol $L^{-1}$ for formate) at days 36 and 50, respectively.

*Constant sulfate/substrate concentration*
: If sulfate/substrate was completely turned over between sampling time points the measured concentration would always be at a similar level, since only the last added amounts would be detected. It is possible that this state is only reached after a lag phase, where accumulation of substrates, and especially sulfate, is seen, before sulfate/substrate levels stabilise or decrease.

*Intermediate sulfate/substrate profile*
: If only a fraction of the added sulfate or substrate is turned over, an increase in their concentrations would be detected, but less than in the total accumulation scenario.

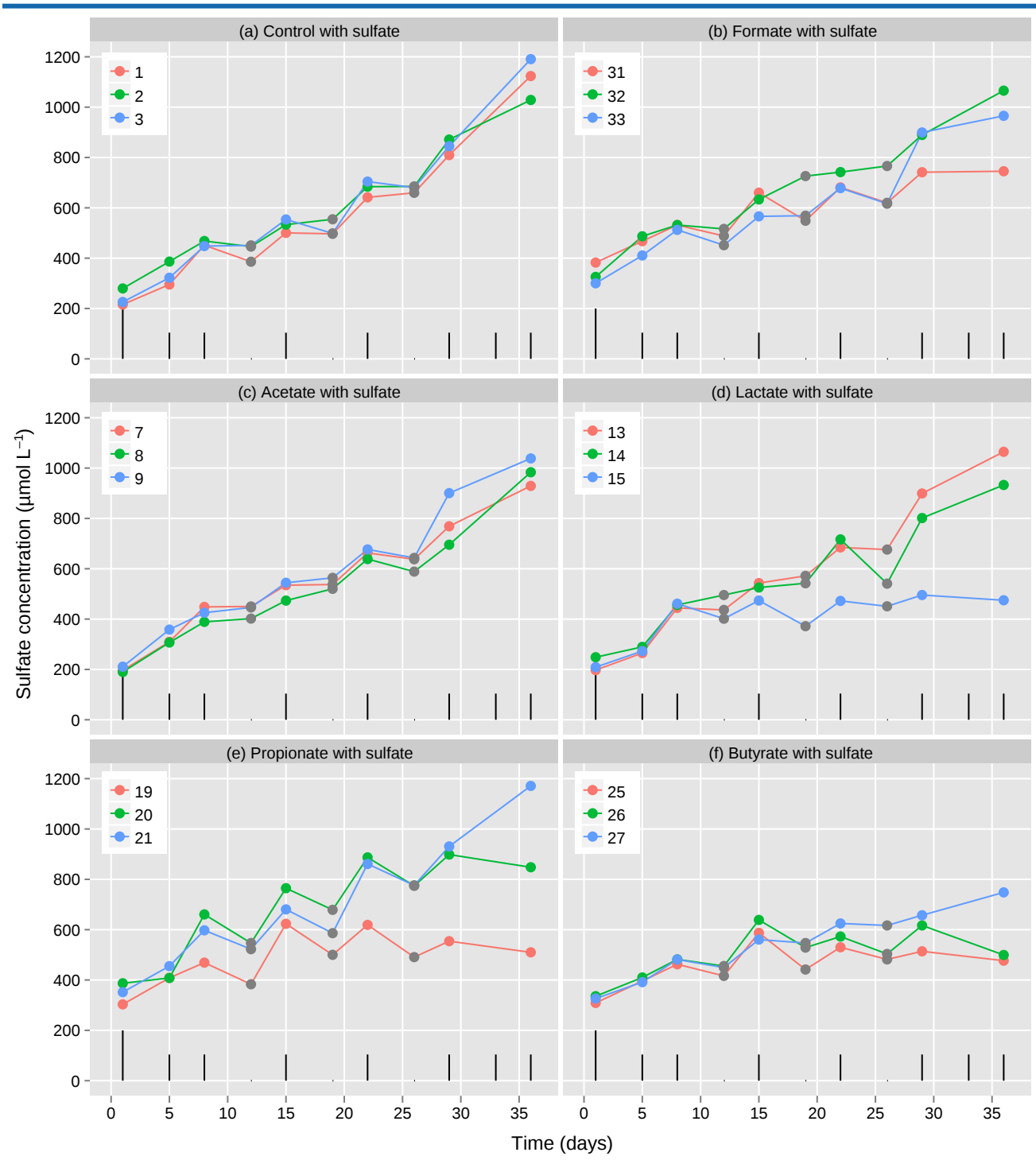


**Figure 3.15** Sulfate concentration profiles of all sulfate-amended biological triplicates from day 1 to 36. Microcosm numbers are given in each subplot. Black bars indicate amounts of sulfate added (200 µmol $L^{-1}$ at day 1, followed by 104 µmol $L^{-1}$ at later time points). Grey dots indicate days where no sulfate was added.

No substrate accumulation was observed in any microcosm, meaning that they were completely turned over between every sampling time point (Figure B.3 to Figure B.7). One exception was microcosm 30, where an increase in butyrate concentration was observed at day 36 (Figure B.7f). In contrast, the sulfate turnover results were very heterogeneous. No sulfate-amended microcosm shows complete sulfate turnover, however, they show different accumulation patterns. In the control incubations supplemented only with sulfate no turnover of sulfate was observed (Figure 3.15a). Minimal sulfate turnover was observed in the incubations supplemented with acetate or formate (Figure 3.15b, c). Compared to the

controls, considerable sulfate turnover was observed in some of the propionate- and lactate-amended microcosms (Figure 3.15d, e). The lowest sulfate concentrations at day 36 were observed in butyrate-amended slurries (Figure 3.15f). Looking at measured time points where no sulfate was added (days 12, 19, and 26; grey dots) gave an indication to whether sulfate was actively turned over. Again on average, no or only small decreases in sulfate concentrations were detected in control, formate-, acetate-, and lactate-amended microcosms. Butyrate- and especially propionate-amended microcosms showed clear drops in sulfate concentrations at these time points.

When sulfate turnover profiles for each microcosm were examined a high variance within replicates became visible. Only control and acetate-amended replicates showed similar responses, whereas the other sulfate-amended microcosms showed considerable differences. Taking overall amounts of sulfate accumulation and changes between sulfate-amended and not amended time points into account it can be said that the following slurries showed considerable sulfate turnover: 1 of 3 formate-, 1 of 3 lactate-, 2 of 3 propionate- and 3 of 3 butyrate-amended microcosms. The third propionate-amended slurry (number 21) showed sulfate concentration decreases at no-sulfate-added time points, however, sulfate considerable accumulated over time.

Small amounts of endogenous sulfate where detected in all microcosms, where no sulfate was added, at day 1 (24 ± 6 µmol $L^{-1}$, 26 % RSD). In most of these microcosms, sulfate was not detectable any more after several weeks (it was detected in all controls and 2 out of 3 acetate microcosm until day 36). In butyrate microcosms this trace amounts were completely turned over within the first 5 days (Table B.1).

### 3.2.3. 16S rRNA qPCR targeting *Desulfosporosinus*

Due to time constraints, only one sulfate-amended microcosm plus an appropriate control were analysed with the newly compiled sample extraction and qPCR pipeline (Section 3.1). First insights into the response of the *Desulfosporosinus* populations in single-substrate incubations were gained. Additionally, the suitability of the evaluated protocol to analyse this kind of sample series was verified. According to the capillary electrophoresis results, butyrate plus sulfate-based slurries showed the highest potential for sulfate reduction, so microcosms 25 (amended with butyrate and sulfate) and 29 (amended with butyrate but not sulfate) were selected for this part.

The extraction batch for this analysis consisted of two time point zero samples, and the soil slurry samples from days 2, 9, 16, and 30 of the incubations 25 and 29. All samples were ground in liquid nitrogen and approximately 300 mg of frozen soil were used to extract nucleic acids with the final CTAB-based protocol. The complete extract was further purified with the OneStep™ PCR Inhibitor Removal Kit. DNase and RNase treatment was done with 85 µL each (total extract volume was 200 µL), followed by quantification, reverse transcription with the SuperScript™ III First-Strand Synthesis System for RT-PCR and analysis with qPCR (the final protocol used is also shown in Figure A.1).

To observe and exclude alterations of the qPCR results by introduction of exogenous DNA (or RNA) by any of the applied methods, NTCs were done at the following steps: nucleic acids extraction and purification, nuclease treatments, reversion transcription and qPCR. Furthermore, an overall NTC was done (labelled X): nucleic acids extraction was done without a soil sample and this "extract" was then applied to each step of the protocol in parallel to the real samples. DNA aliquots were diluted and a total of 5 ng gDNA was used in the qPCR assay. In the RT-qPCR approach, 1 ng RNA was applied to the first strand synthesis. 5 µL of a 10-fold dilution of this cDNA solution was then quantified (which equals to 0.025 ng of reverse transcribed RNA). Dilution series of the DNA and cDNA samples from microcosm 25 (day 9) were also analysed, as already described in Section 3.1.

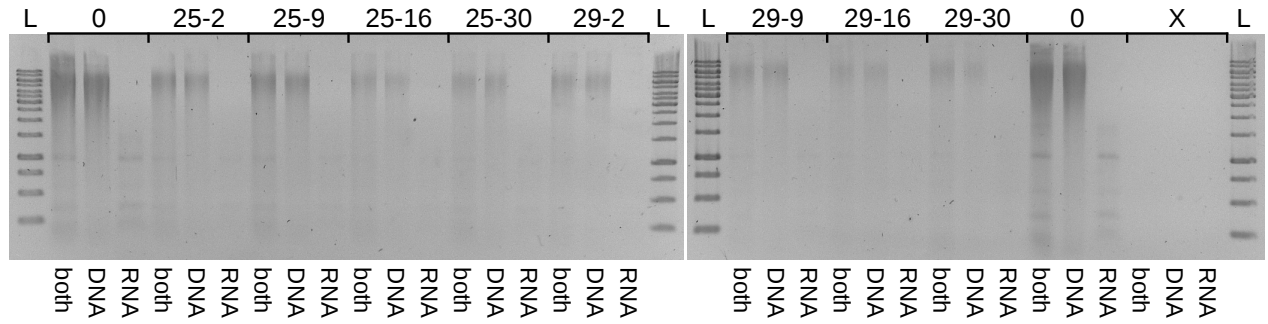


**Figure 3.16** Agarose gel electrophoresis of samples before and after nuclease treatments (1 % agarose, 120 V, 50 min). *L* GeneRuler™ 1 kb DNA Ladder; *X* negative control; *0* time point zero samples; *##-#* slurry samples (number and incubation day); *both* total nucleic acids extract after purification; *DNA* DNA aliquot after RNase treatment; *RNA* RNA aliquot after DNase treatment.

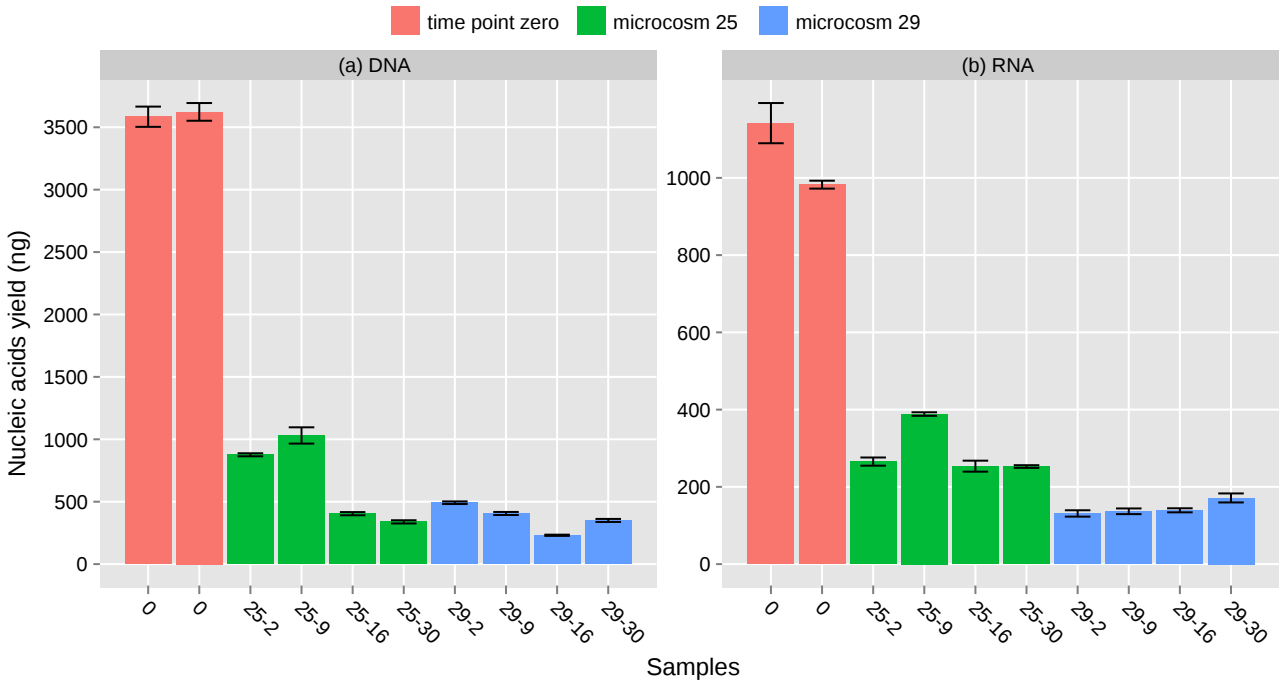


**Figure 3.17** Quantification of DNA **(a)** and RNA **(b)** samples done with the Quant-iT™ PicoGreen® dsDNA and RiboGreen® RNA kits. Standard curve quality indicators: $R^2$ = 0.997 and 0.999, respectively. Error bars are standard deviations of technical replicates in the Quant-iT™ assays (n = 3).

Total nucleic acids extracts and also purified DNA and RNA aliquots were analysed with gel electrophoresis (Figure 3.16). rRNA bands were observed in some of the samples, especially in the time point zero samples. No DNA was visible in the purified RNA samples. The quality of the DNA aliquots was also analysed with a NanoDrop spectrophotometer. The time point zero samples showed typical wavelength-absorbance curves with 260:280 ratios of 1.69 and 1.71. The 260:280 ratios of the slurry samples were between 1.41 and 1.68 (1.51 ± 0.08, 5 % RSD). All DNA and RNA samples were quantified with Invitrogen's Quant-iT™ assays (Figure 3.17). The yield of time point zero samples was much higher than those of samples from later time points, which corresponds with the agarose gel electrophoresis (Figure 3.16) and NanoDrop results. Approximately the same amount of sample was used in all extractions. But since the slurries consisted of 30 g soil and 60 mL sterilised fen water and the time point zero samples were only soil, and the sample amounts were determined by wet weight and not dry weight, a higher yield was to be expected in time point zero samples. On average, the DNA

and RNA yield was higher in samples which were incubated with sulfate than in those without sulfate supplementation.

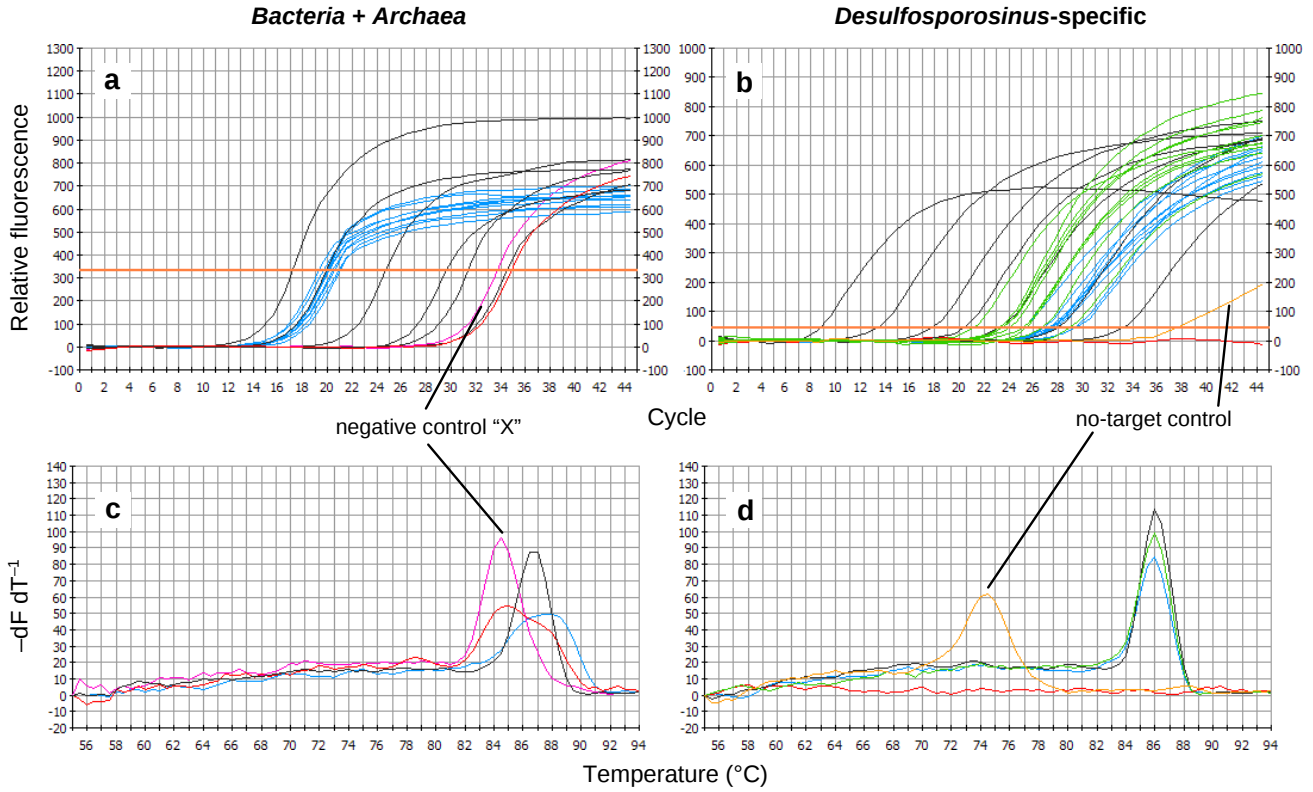


**Figure 3.18** Representative 16S rRNA qPCR results. Traces of standard dilution series (black), qPCR NTCs (red), and DNA samples (blue) shown. The horizontal, orange line indicates the threshold used for determination of quantification cycles. –dF $dT^{-1}$ is the negative first derivative of fluorescence versus temperature. **(a)** Traces and **(c)** melt curves of qPCR assay targeting total *Bacteria* & *Archaea*. The complete-protocol NTC X is also plotted in pink. No cDNA results are plotted because of inconsistent results. qPCR standard curve quality indicators: $R^2$ = 0.990, PCR efficiency = 90.9 %. Standard curve dilutions $10^{-9}$ not shown. **(b)** Traces and **(d)** melt curves of *Desulfosporosinus*-specific 16S rRNA qPCR. cDNA samples are plotted as green traces. The no-target control is also plotted. qPCR standard curve quality indicators: $R^2$ = 0.994, PCR efficiency = 80.0 %. Standard curve dilutions $10^{-10}$ and $10^{-11}$ not shown.

All samples and controls were applied to the qPCR and RT-qPCR assays targeting total *Bacteria* & *Archaea* and *Desulfosporosinus* (Figure 3.18). No problems were encountered when DNA aliquots were analysed (blue traces). Despite the discouraging results from the *Bacteria* & *Archaea* RT-qPCR evaluation (Section 3.1.5.2), cDNA samples were analysed with both assays (green traces). In all samples, more 16S rRNA copies were detected in the assay targeting *Desulfosporosinus* than in the assay targeting total *Bacteria* & *Archaea* (200–1700 %). Ten times stronger diluted cDNA samples were also analysed with the *Bacteria* & *Archaea* RT-qPCR, which resulted in 16S rRNA copy numbers that were sometimes higher and sometimes lower than those of the less diluted samples. Because of this inconsistencies, the *Bacteria* & *Archaea* RT-qPCR data was not included in any further analysis. In addition, RNA aliquots were tested for endogenous DNA contaminations. They were quantified in the same range as the NTCs, with the exception of one (out of two) technical replicates of the overall NTC X, which had an lower $C_q$, but was considered an outlier. No additional investigation was done, since RT-qPCR data from the qPCR assay targeting *Bacteria* & *Archaea* was not used in the final analysis and no contamination with *Desulfosporosinus*-DNA was detected. The overall DNA negative control X had a higher $C_q$ than the qPCR's NTC (pink trace in Figure 3.18a). By comparison with the per-step controls, it could be determined that the RNase treatment step introduced trace amounts of foreign DNA, none of which was *Desulfosporosinus*-specific DNA. The number of 16S rRNA genes introduced

was so low compared to the real samples (0.02 % compared to the lowest sample copy number), that this contamination was considered to be of no relevance. The no-target control in the *Desulfosporosinus*-specific qPCR was amplified at late cycles (orange trace in Figure 3.18b). When looking at the corresponding melt curve analysis, it can be seen that no full length and therefore unspecific PCR product was amplified. All other negative controls in this assay, including selected not-reverse-transcribed RNA samples and reverse transcription reactions with RNA but without the enzyme, were not amplified at all.

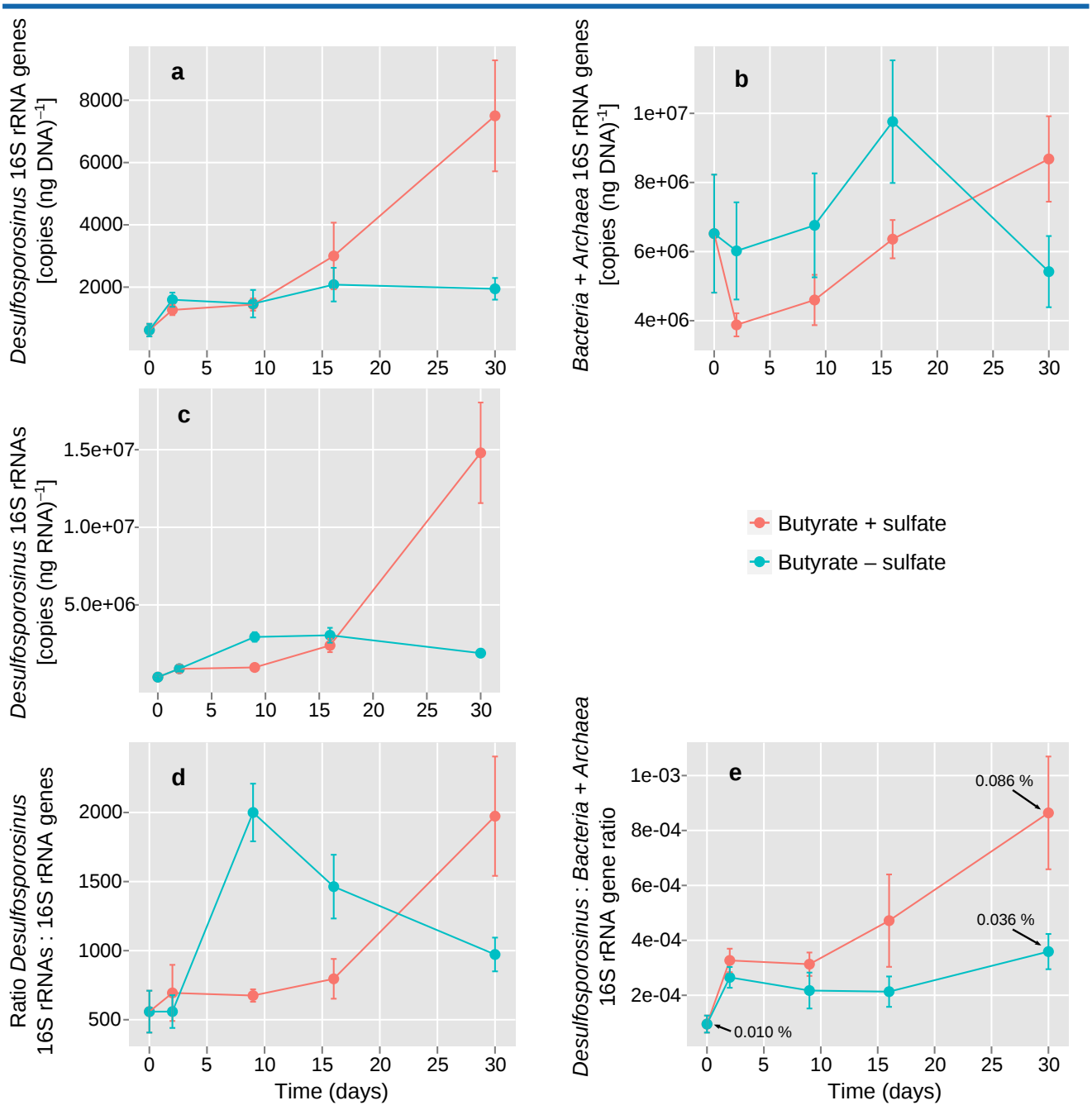


**Figure 3.19** **(a–c)** Total *Bacteria* & *Archaea* and *Desulfosporosinus* 16S rRNA gene and reverse transcribed 16S rRNA copy numbers from two butyrate-supplemented incubations are plotted. Sulfate-amended incubation microcosm 25 is shown in red. Control microcosms 29 (without sulfate supplementation) is displayed as blue. qPCR quality indicators are already described in Figure 3.18. Error bars are standard deviations of technical replicates in the qPCR assay. **(d)** Ratios of *Desulfosporosinus* 16S rRNA copies against 16S rRNA gene copies. Error bars are standard deviations of technical replicates in the RT-qPCR assay. **(e)** Ratio of 16S rRNA gene copy numbers of *Desulfosporosinus* versus total *Bacteria* & *Archaea*. Error bars are standard deviations of technical replicates in the qPCR assay with *Desulfosporosinus*-specific primers.

The qPCR results were normalised against starting amounts of DNA/RNA and plotted. In the microcosm without sulfate, the number of total *Bacteria & Archaea* 16S rRNA genes fluctuates over time. In the sulfate-supplemented microcosm the copy numbers increase over time, except for the first time point (Figure 3.19b). Analysis with the *Desulfosporosinus* qPCR showed only minimal increases in 16S rRNA genes and transcripts in the control, while a considerable increase was observed in the sulfate-amended slurry (Figure 3.19a, c). The same results were observed when *Desulfosporosinus* 16S rRNA genes were normalised against the number of total *Bacteria & Archaea* 16S rRNA genes (Figure 3.19e). The ratio of *Desulfosporosinus* 16S rRNA transcript copy numbers versus 16S rRNA *gene* copy numbers is plotted in Figure 3.19d, which represents the relative number of 16S rRNAs per *Desulfosporosinus* 16S rRNA gene operon. In the sulfate- and butyrate-supplemented microcosm an exponential increase was detected between time points 9 and 30. In the control slurry a faster increase was observed, but after the peak at day 9, the ratio decreased again.

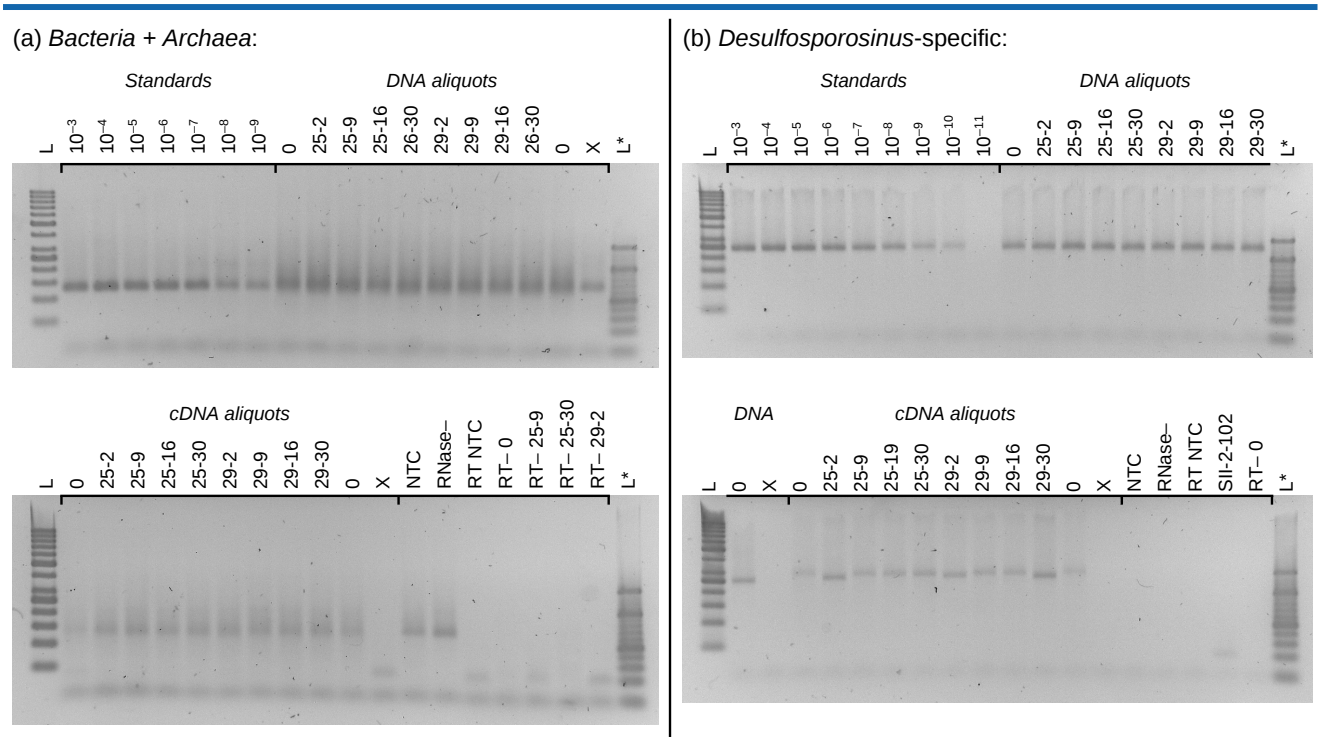


**Figure 3.20** Agarose gel electrophoresis of qPCR products (2.5 % agarose, 120 V, 60 min). *L* GeneRuler™ 50 bp DNA Ladder; *L** O'RangeRuler™ 20 bp DNA Ladder; *$10^{-3}$–$10^{-11}$* Standard dilution series (see Section A.8); *0* time point zero samples; *##-#* slurry samples (number and incubation day); *X* overall negative (no-template) control; *NTC* no-template control for qPCR assay; *RNase–* negative (no-template) control from RNase step; *RT NTC* no-template control of the reverse transcription step; *RT– 0/##-#* same as cDNA samples, but no reverse transcriptase was added at the RT step; *SII-2-102* no-target control. 5 ng of DNA or 0.025 ng of reverse transcribed RNA were used as templates (*DNA* and *cDNA* respectively). qPCR quality indicators are already described in Figure 3.18. **(a)** Results from the total *Bacteria & Archaea* qPCR; PCR product bands are at approximately 140 bp (*in silico* size of standard-series PCR products: 123 bp). **(b)** Results from the *Desulfosporosinus*-specific qPCR; PCR product bands are at approximately 250 bp (*in silico* size of standard-series PCR products: 236 bp).

Finally, the qPCR products were analysed using gel electrophoresis. Figure 3.20a and b shows the results from the 16S rRNA qPCR assays targeting total *Bacteria & Archaea* and *Desulfosporosinus*, respectively, which correspond with the data obtained from the qPCR itself. Standards and samples all showed the same band. In the *Desulfosporosinus*-specific assay, the lowest standard and the negative controls only showed primer dimer bands at the bottom of the gels. Negative controls from the *Bacteria & Archaea* assay also showed the same bands as the samples (expect cDNA negative controls), which coincides with the qPCR results. With the 16S rRNA qPCR assay targeting *Bacteria & Archaea*

the bands of the PCR products are not as clear as with the assay targeting *Desulfosporosinus*, especially with the samples, which can be explained by PCR products varying in size. The same was already indicated by the melt curve analysis shown in Figure 3.18c and d, where the melt peak of the *Desulfosporosinus*-specific assay is more defined than those of samples analysed with the *Bacteria* & *Archaea* assay. It must be kept in mind, that varying PCR product sizes introduce a bias into the quantification.

## 3.3. Short-term peat soil incubations

Sequential numbers were assigned to all soil slurries (see Table B.1) and are given in parentheses throughout this section for the sake of completeness. These microcosms should not be confused with the microcosms from the long-term incubations, even though they are numbered in the same pattern.

### 3.3.1. Sulfate and substrate turnover

In contrast to the long-term incubation experiment, where capillary electrophoresis was primarily used as a screening method, the results of this experiment were included in the calculation of sulfate reduction rates. Therefore the range of the standard series was expanded down to 6.25 µmol $L^{-1}$ and more time was invested into proofreading peaks.

120 µmol $L^{-1}$ of substrates were turned over within 2–3 days (1–2 days in the case of formate) in all slurries; they were slightly faster turned over in sulfate-amended microcosms (Figure B.8 to Figure B.13). Minimal net decrease in sulfate concentration was observed in all sulfate-amended microcosms (200 µmol $L^{-1}$ were supplemented at day 0), including the controls without additional substrates (Figure 3.21). In the sulfate- and lactate-amended microcosms, no increase in sulfate concentrations was measured on the last day (Figure 3.21d), as in the other sulfate-amended incubations (which was caused by addition of sulfate). The most parsimonious explanation is that the addition of the sulfate was forgotten at day 5 with these slurries.

Like in the long-term incubation experiment, small amounts of endogenous sulfate where detected at day 0 in all microcosms, where no sulfate was added (17 ± 6 µmol $L^{-1}$, 36 % RSD). Within the six days of incubation, no sulfate was turned over, with the exception of one acetate-amended microcosms (number 11, Figure B.10e), which also showed coinciding acetate accumulation after the sulfate level dropped below the detection limit.

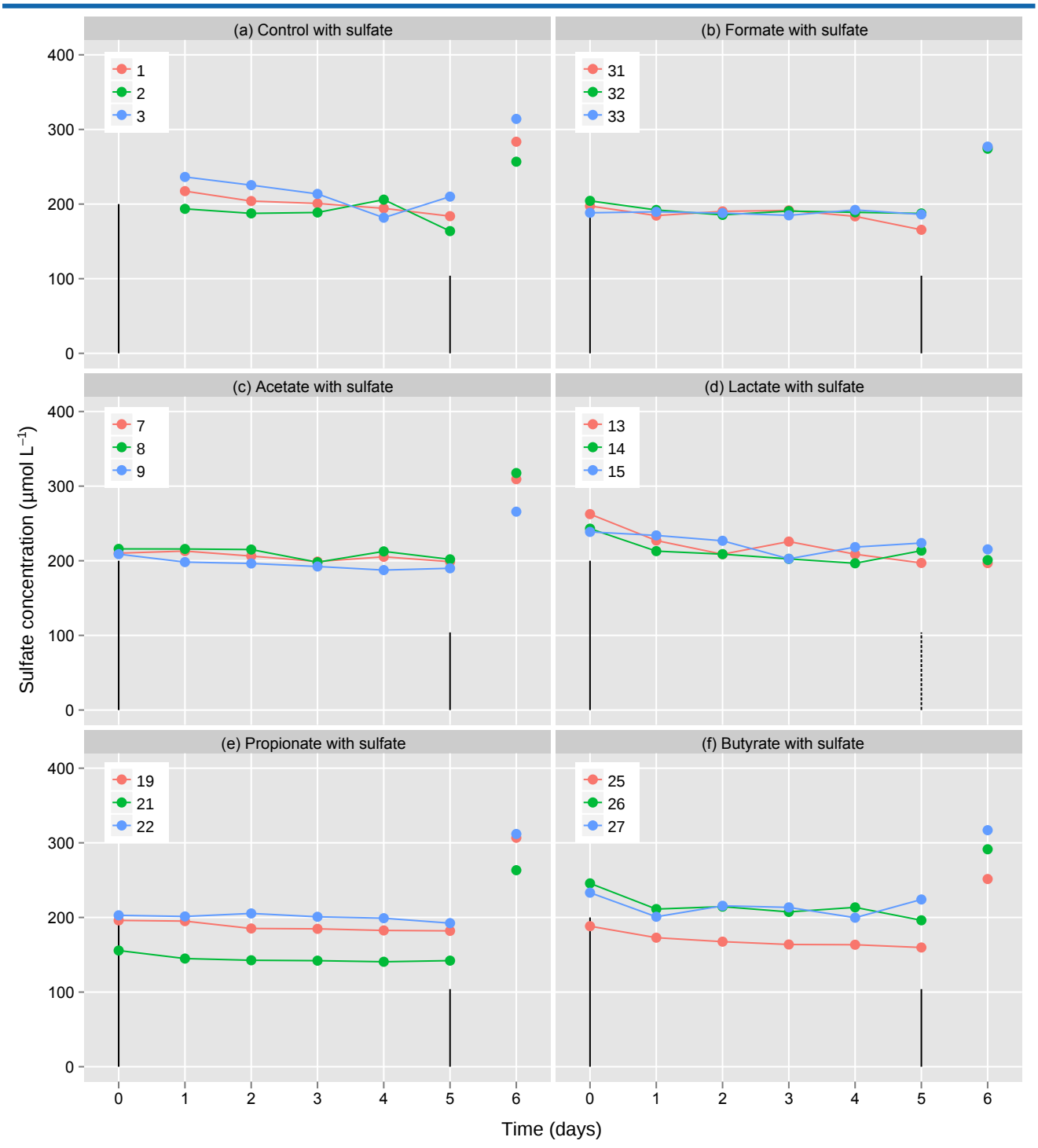


**Figure 3.21** Sulfate concentration profiles of all sulfate-amended biological triplicates. Microcosm numbers are given in each subplot. Black bars indicate amounts of sulfate added (200 and 104 µmol $L^{-1}$). No lines between days 5 and 6 are plotted, because sulfate concentration increases were caused by addition of external sulfate, and not by an internal factor. All sulfate peaks at day 0 in the sulfate-amended controls were outliers and are not plotted. Normalisation against the internal standard was omitted, because all samples from the same microcosm were always measured in the same measurement batch.

### 3.3.2. Sulfate reduction rates

Based on unpublished data from $^{35}S$ radiotracer measurements done by Dr. Knorr (University of Bayreuth) and sulfate concentrations from day 6 done by the author of this thesis, sulfate reduction rates could be calculated (Figure 3.22). In all microcosms, sulfate reduction was measured. In the for-

mate- and acetate-amended incubations only minimal difference was seen between the slurries with and without sulfate supplementation. With the other substrates, a higher range of sulfate reduction rates was observed in slurries with additional sulfate than in those without, even in the controls. However, a high variance in response of slurries with sulfate was observed.

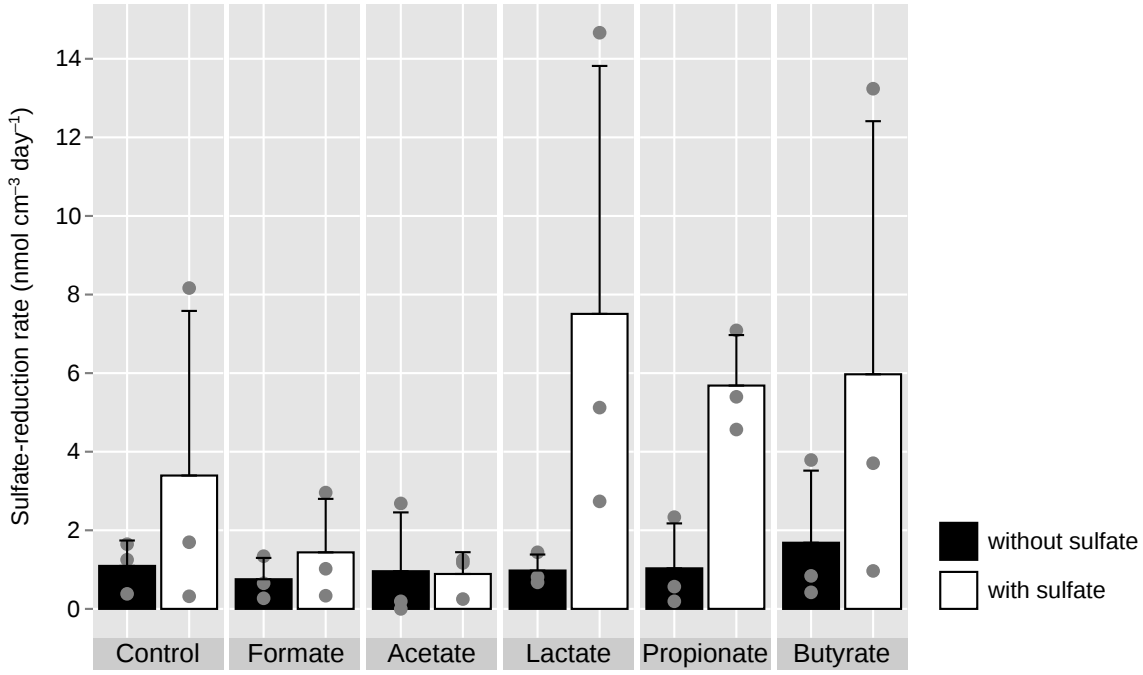


**Figure 3.22** Sulfate reductions rates in the short-term incubations at day 6. Grey dots are results from biological triplicates and error bars are standard deviations of these replicates.

### 3.3.3. Sensitivity assessment of capillary electrophoresis measurements

All standard curve data from this experiment's capillary electrophoresis measurements was used to evaluate the sensitivity of this method. For quantification of sulfate and substrate concentrations a standard curve was measured twice in each sampling session. In total, 26 complete standard curves for sulfate and each substrate were measured. For this analysis all the measurements were combined into six standard curves (one for sulfate and one per substrate). $R^2$s of the six standard curves ranged from 0.994 to 0.995. In comparison, while analysing single session standard curves the highest $R^2$ observed was 1.000, the lowest 0.996.

A more detailed analysis of each part of the standard curves' peak areas is shown in Figure 3.23. With decreasing concentration the relative standard deviation rises. The RSD of the 100–400 µmol $L^{-1}$ standard dilutions is approximately 5–6 %, while the RSD at 6.25 µmol $L^{-1}$ dilutions increases to 19–25 %. Within one standard dilution, sulfate and substrate peak areas had similar relative standard deviations. It was tested if the internal standard (always 190 µmol $L^{-1}$) was affected by what dilution of the standard curve was measured, but its peak area's RSD fluctuated between 5 % and 8 % with no correlation to the dilutions.

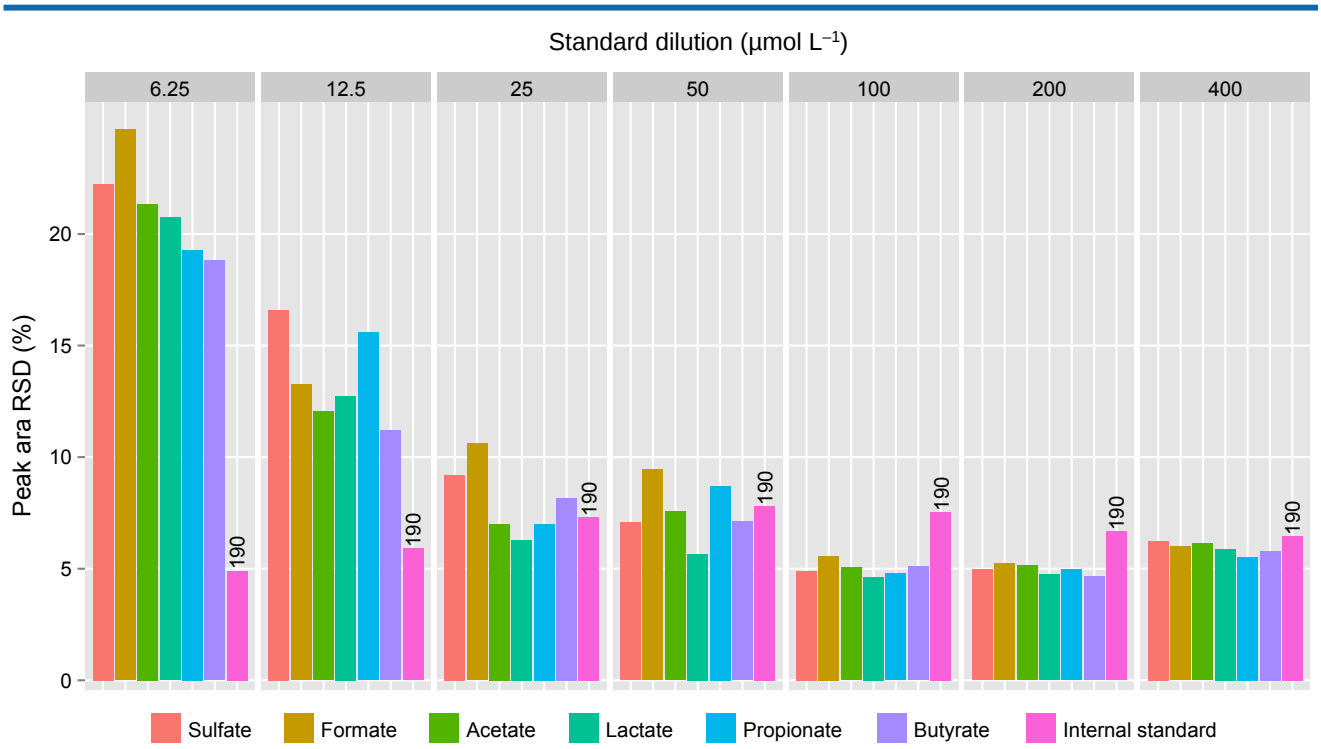


**Figure 3.23** Peak area RSDs of each standard curve dilution (µmol $L^{-1}$), separated by substrate. The internal standard always had the same concentration (190 µmol $L^{-1}$), but for this plot, the internal standard peak areas were grouped according to the standard curve dilution in which they were measured.

## 3.4. Enrichment of sulfate-reducing microorganisms

The goal of this experiments was to enrich and cultivate SRMs (especially *Desulfosporosinus*) from the long-term incubation experiment, which could give additional insights into biological processes, and the microorganisms responsible for those processes, in the Schlöppnerbrunnen fens. Sulfate additions were higher than in the long- and short-term incubations (5 mmol $L^{-1}$) and substrates were added in concentrations that corresponded to 100 % mineralisation to $CO_2$ by sulfate reduction (see Section 2.6.3)

Substrate concentration profiles of the successful enrichment cultivations are shown in Figure 3.24. The same standard series as in the incubation measurements was used, but the samples were diluted 20-fold before preparation for capillary electrophoresis measurement. Determination of sulfate and substrate concentrations of all samples was not done in replicates. Furthermore, the final volume in the cultivation tubes changed due to sampling and sulfate/substrate additions. Therefore the concentration profiles may not be as precise as in the incubation experiments, but trends and distinctive changes in concentrations can be used to reveal sulfate and substrate turnover.

The enrichment with sterilised soil as substrate showed a slow, but constant, turnover of sulfate. After each fresh inoculation (= addition of new soil) acetate accumulation can be seen in the beginning, which was later turned over again (Figure 3.24a). Due to the complex substrate (soil) of this enrichment culture, it will likely be impossible to gain a pure culture, but nevertheless, the enrichment could be used for culture-independent experiments. Sulfate concentrations often were higher than the added 5 mmol $L^{-1}$ at the first time points for each enrichment step, which can be explained by carry-over from the previous tube/bottle.

In the beginning of the enrichment experiment, the fastest sulfate turnover was seen in the formate-based enrichment culture, where sulfate and almost all formate was depleted in three weeks.

From the concentration differences between the first and last time points of the first two enrichment steps, it was calculated that 91 % and 95 % of formate turnover could be attributed to sulfate reduction, respectively (under the assumption that sulfate reduction was the only active sulfate-related process). After the third inoculation the culture showed no more activity. Only after trace amounts of yeast extract (10 mg $L^{-1}$) were added on day 183, a response of the culture could be observed, but sulfate turnover was much slower than in the first two enrichment steps and sulfate reduction could only account for 85 % of formate turnover (Figure 3.24b).

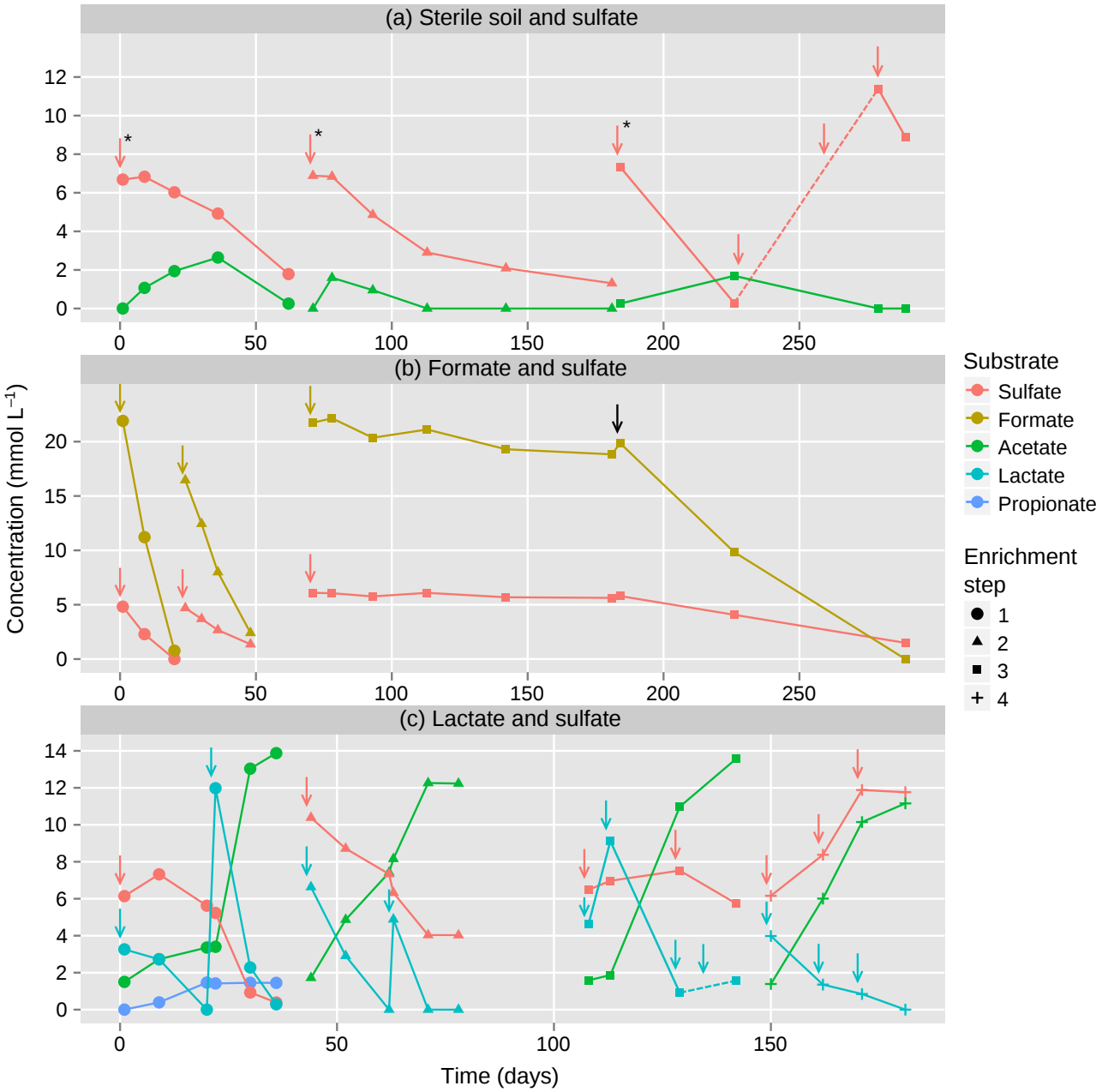


**Figure 3.24** Sulfate and substrate turnover profiles in enrichment cultures with sulfate as electron acceptor and sterile soil (a), formate (b), or lactate (c) as electron donors. Inoculations of new tubes (enrichment steps) are indicated by different and not connected symbols. Coloured arrows mark the time points when sulfate/substrates were added (normally one day before samples for capillary electrophoresis measurement were taken). The black arrow marks the addition of yeast extract. Asterisks mark addition of sterilised soil (and sulfate). Dotted lines are drawn when sulfate/substrates where added but no concentration measurements of the following day were done.

The lactate-based enrichment always showed formation of acetate. In case of complete oxidation of lactate by sulfate reduction, 5 mmol $L^{-1}$ of sulfate with 3.3 mmol $L^{-1}$ of lactate (ratio 1:0.66) should both be completely turned over to hydrogen sulfide and $CO_2$ (see Table 1.2), but supplemented lactate was always depleted before sulfate. This indicates that lactate is incompletely oxidised to acetate. Therefore lactate was added a second time and more sulfate turnover was observed. In the third enrichment step the culture showed a short lag phase, but at the third measurement time point (day 129) considerable sulfate turnover was seen (sulfate was added on the day before, but compared to the last measured time point the concentration did not increase much, so sulfate had to be turned over in between the two time points) and lactate, which was also added on the day before, was already nearly depleted. After the forth inoculation, 5 mmol $L^{-1}$ sulfate and 3.3 mmol $L^{-1}$ lactate were added multiple times. Between the first and third measured time point of this enrichment step, 10 mmol $L^{-1}$ of sulfate were added, but the concentration only increased by 6 mmol $L^{-1}$, meaning 4 mmol $L^{-1}$ were turned over. No turnover is seen after that, but lactate levels were already very low (Figure 3.24c). From day 129 on, a detectable increase in optical density could be seen in this enrichment lineage and sulfate/substrate concentration measurements were only done irregularly from that time point on.

In the tenth enrichment step of the lactate-based enrichment, sulfate and substrate turnover was measured at four consecutive days. 1.6 mmol $L^{-1}$ of sulfate and 3.9 mmol $L^{-1}$ of lactate were turned over while 3.7 mmol $L^{-1}$ of acetate was produced. Sulfate reduction could therefore account for 83 % of all lactate turnover, if lactate would be incompletely oxidised to acetate. Growth of this enrichment step was also monitored by optical density measurements (Figure 3.25). After a two days lag phase, exponential growth was seen. After 11–13 days the culture reached a stationary phase, but after addition of sulfate and lactate, cell division continued much faster. Three days later the culture stagnated and did not start growing again even after addition of new sulfate and lactate.

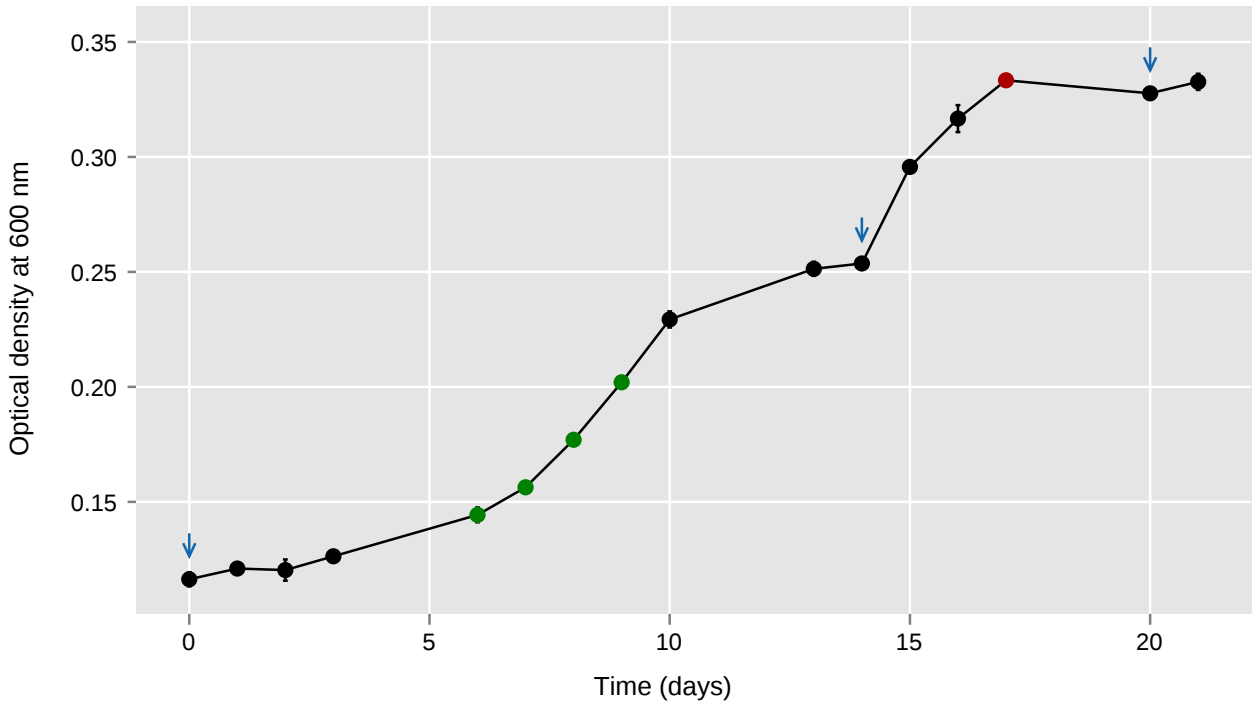


**Figure 3.25** Optical density curve of the lactate-based enrichment during the tenth enrichment step. Measurements were done at 600 nm and blanked against air. Error bars are standard deviations of technical replicates (n = 3). Arrows mark additions of 5 mmol $L^{-1}$ of sulfate and 10 mmol $L^{-1}$ of lactate. Days where sulfate and substrate concentrations where additionally measured with capillary electrophoresis are marked green (days 6–9). FISH was done with samples taken at day 17 (red dot), see Figure 3.27b–d.

Enrichment attempts with acetate showed sulfate turnover in the first enrichment step, but after inoculation no more activity could be detected (Figure B.14a). Propionate- and butyrate-based enrichments showed sulfate and substrate turnover and production of acetate for two enrichment steps but then stagnated after the third inoculation, too (Figure B.14b, c).

*Firmicutes*, but no *Deltaproteobacteria*, could be detected in the first enrichment step in lactate- and butyrate-based cultures with the applied probe mixtures (Figure 3.27a). No difference could be seen in hybridisation behaviour between PFA- and ethanol-fixed samples in first enrichment step samples (data not shown). At the tenth enrichment step of the lactate-based enrichment, however, no more *Firmicutes* could be detected (Figure 3.27b). Most of the cells in this enrichment were *Deltaproteobacteria*, but some larger, rod-shaped, not identified *Bacteria* were seen as well (Figure 3.27c). The same enrichment was also tested for the presence of *Archaea* but non could be detected with the used probe (Figure 3.27d).

Neither *Firmicutes* nor *Deltaproteobacteria* were visible in the first enrichment step of the formate-based enrichment (Figure 3.26a), but small rod-shaped cells could be seen with DAPI-staining (Figure 3.26b). A sample from the third enrichment step was analysed as well, but again no *Deltaproteobacteria*, *Firmicutes* or *Archaea* could be detected with the applied probe mixtures (data not shown). Cells could be seen with light microscopy (data not shown), but the *Bacteria* probe did not gave a signal. This could be explained by the fact, that the culture was most likely in the stationary phase when the sample was taken.

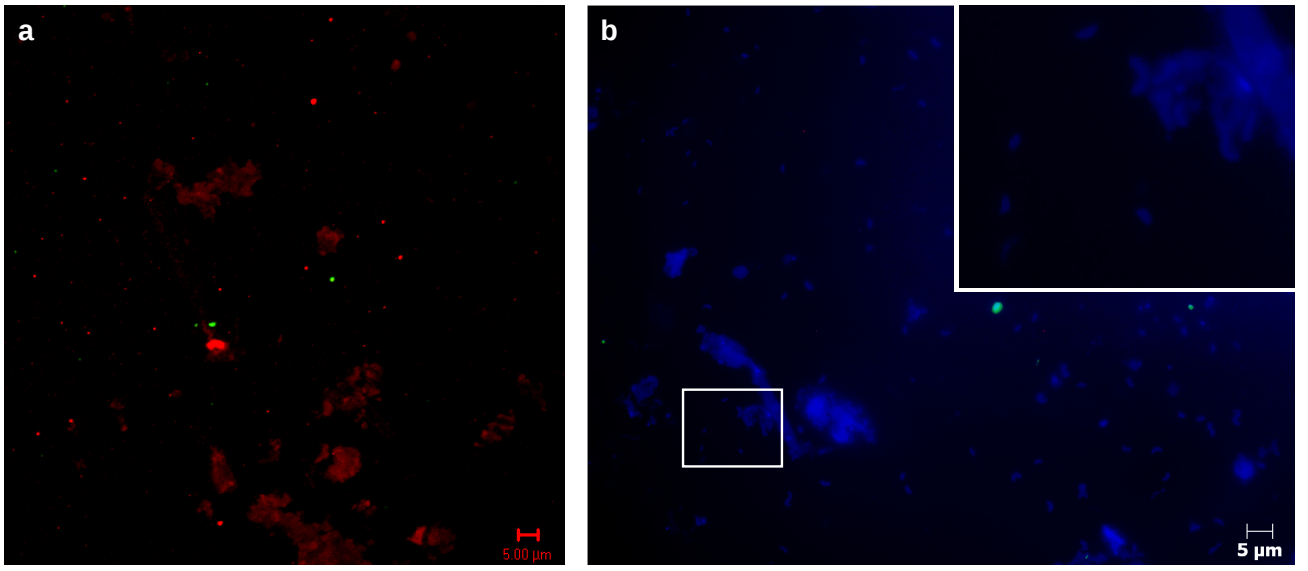


**Figure 3.26** Fluorescence *in situ* hybridization images of a PFA-fixed formate-based enrichment sample from the first enrichment step. Bars are 5 µm. The sample was also hybridised with the NONEUB probe to exclude unspecific signals (not shown). **(a)** Sample with LGC354mix (red) and Delta495mix (green) probes. FISH was done with the help of Andreas Anderluh (Department of Microbial Ecology). **(b)** The same hybridisation as in (b), but DAPI-stained. Magnified section is marked with a white box.

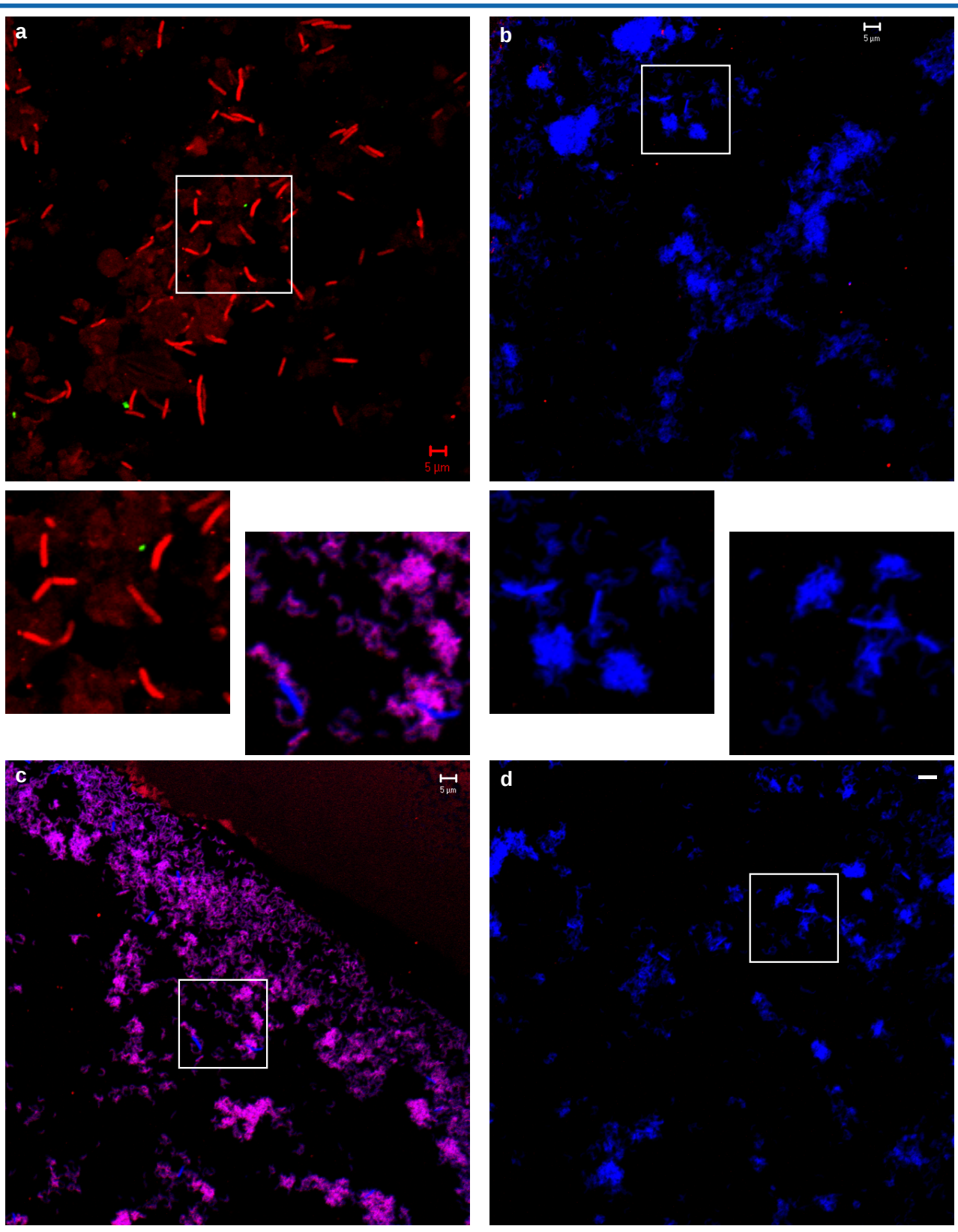


**Figure 3.27** Fluorescence *in situ* hybridization images of the lactate-based enrichment. Bars are 5 µm. Magnified sections are marked with white boxes. The samples were also hybridised with the NONEUB probe to exclude unspecific signals (not shown). **(a)** PFA-fixed sample from the first enrichment step with LGC354mix (red) and DELTA495mix (green) probes. FISH was done with the help of Andreas Anderluh (Department of Microbial Ecology). **(b–d)** Ethanol-fixed sample from the tenth enrichment step with (b) LGC354mix, (c) DELTA495mix, (d) ARCH915 (red) and EUB338mix (blue) probes. The combination of the DELTA495mix and EUB338mix probes results in a pink signal in (c). The structure in the upper right corner of (c) is autofluorescence from the polytetrafluoroethylene coating on the wells boundaries.

# Chapter 4. Discussion



## 4.1. Optimisation of nucleic acids extraction and qPCR analysis conditions for Schlöppnerbrunnen soil slurries

Nucleic acids from the total microbial diversity in an environmental sample can not be completely recovered with only one single extraction protocol. Additionally, every type of environment is different and therefore requires customized methodology. Soil is a complex and diverse environment which makes it even more difficult to extract DNA and RNA. Two commons challenges are the separation of nucleic acids from coprecipitants, like humic acids, and the fact that microbial cells differ (e.g. gram-positive vs gram-negative, endospores) and therefore cannot be lysed with every method at the same efficiency (Zhou *et al.*, 1996, Bürgmann *et al.*, 2001; Robe *et al.*, 2003; Saleh-Lakha *et al.*, 2005; Delmont *et al.*, 2011). However, this study specifically targets SRMs and especially *Desulfosporosinus*, therefore the extraction bias was only tested in regard to *Desulfosporosinus* by quantifing its 16S rRNA gene copies (Figure 3.4). Further evaluation of extraction efficiencies could have been done by adding known amounts of cultivated microbial (SRM) cells to the environmental samples, followed by DNA or RNA quantification, or analysis of cell lysis by microscopy (e.g. Moré *et al.*, 1994, Alm & Stahl, 2000).

### 4.1.1. Extraction of nucleic acids

The first evaluation step was the selection of an appropriate and efficient protocol for coextraction of DNA and RNA. It is possible to extract DNA and RNA with two separate protocols, which could then be separately optimised for either extraction of DNA or RNA. However, this approach also has some disadvantages, therefore coextraction was done, followed by separation into DNA and RNA. The major disadvantage of separate extractions is that every nucleic acids extraction method has a bias in extraction efficiency and specifity, especially if different protocols and parameters are used (see above). Since both the DNA and RNA aliquots were analysed with (RT-)qPCR and the results directly compared, it was important to keep this bias to a minimum within one sample. Practical reasons for choosing the coextraction approach were that the amount of sample was limited and that separate extractions are more time consuming and expensive. The TNS-based method (Lueders *et al.*, 2004), used in the study which primed this research (Pester *et al.*, 2010), was compared to a similar CTAB-based method (Griffiths *et al.*, 2000). Both are direct lysis methods based on bead beating for mechanical cell lysis, and phenol/chloroform/isoamyl alcohol extraction for chemical lysis and separation of nucleic acids from proteins. The major problem when extracting nucleic acids from soil is the coprecipitation of inhibitory substances, e.g. humic acids (Zhou *et al.*, 1996). The TNS-based method was already adapted by adding an extra potassium acetate precipitation step to remove humic acids (Bodrossy *et al.*, 2006; Pester *et al.*, 2010). The component responsible for purification in the CTAB-based method is CTAB itself. It has been shown that CTAB can partially remove humic acids without any loss of DNA (Zhou *et al.*, 1996). Four different combinations of bead beating speeds and times were additionally tested with the CTAB-based method.

When total nucleic acids extracts were examined with agarose gel electrophoresis, DNA yields and 16S rRNA bands were strongest in the CTAB-based extracts with the highest bead beating settings. However, since 16S rRNAs and 1 kb-long DNA fragments migrate through the gel at the same speed, the intensity of the 16S rRNA bands may be amplified by these DNA fragments and the actual 16S rRNA content of the sample could be lower than it appears on the gel image. (Figure 3.2a). This is supported by the fact, that after separation of RNA from DNA and quantification thereof, the RNA yield turned out to be lowest at this settings (Figure 3.3b). The overall DNA yield, determined with Invitrogen's Quant-iT™ assay, was highest with the CTAB-based method at the highest bead beating settings (Figure 3.3a), but so were humic acids extraction (Figure 3.2b), which coincides with findings of Bürgmann *et al.* (2001). It was reported that increases in bead beating intensity are directly linked, not only to DNA yield, but also to DNA fragmentation (Rajendhran & Gunasekaran, 2008). This leads to the conclusion that RNA may be affected as well and part of the RNA could have been destroyed and subsequently lost, explaining the inverse relationship between RNA yield and bead beating times and speeds. Leite *et al.* (2012) observed that increased bead beating times also resulted in stronger degradation of extracted (fungal) RNA. They suggest that this was caused by endogenous RNases, which in the case of CTAB-based protocol should not be a problem, since bead beating was done directly in Phenol. Other studies, however, reported no effect on rRNA integrity even with long bead beating times (e.g. Zoetendal *et al.*, 1998). It should, however, be noted, that no detectable increases in DNA fragmentation (only in yield) were observed when comparing different bead beating parameters (Figure 3.2a). Analysis with electrophoresis methods that can separate larger DNA fragments, e.g. pulsed field gel electrophoresis, may have revealed differences in fragmentation. The observed DNA fragmentation was within previously reported ranges (Bürgmann *et al.*, 2001), and the majority of DNA fragments were at least ten times larger than the desired qPCR products (123/236 bp). Since the amount of RNA is normally the limiting factor, it was decided to select gentle parameters and accept lower DNA yields instead, which also has the advantage of less coextraction of humic acids.

In terms of DNA and RNA yield, the TNS-based method was inferior to the CTAB-based method. One reason for the superiority in extraction yields of the CTAB-based method could be the bead beating step, i.e. bead beating in PCI and buffer instead of bead beating in buffer followed by PCI extraction (as in the TNS-based method). Phenol deactivates proteins, therefore the contents from lysed cells, e.g. DNases and RNases, can not degrade the nucleic acids. In the TNS-based method the sample had to be split because the potassium acetate precipitation step increased the sample volume and the available microcentrifuge tubes were not large enough for the following washing step with chloroform/isoamyl alcohol (only two thirds can be used in one extraction, the other third has to be separately extracted, see sample 11 in Section 3.1.1). This "duplication" of samples is another disadvantage of the TNS-based method, especially since one extraction yielded less DNA and RNA than the other for unexplained reasons, even though the same sample amounts were used (the remaining thirds of two samples from the same source were pooled). Adapting the protocol by changing volumes of applied solutions could bypass this problem, but would have required another round of evaluations.

Every nucleic acids extraction protocol has a different bias when breaking up microbial cells, depending on the method and its parameters. Bacterial and archaeal cells have a wide range of cell wall types and can not be equally extracted by any one method (Delmont *et al.*, 2011). The primary organisms of interest were *Desulfosporosinus* species, therefore the TNS- and CTAB-based methods were compared with 16S rRNA qPCR assays targeting *Desulfosporosinus*. Most *Desulfosporosinus* species stain gram-negative, but all have the ability to form endospores, which are generally harder to recover than vegetative cells (Kuske *et al.*, 1998). However, only minimal differences were observed between both methods and different bead beating parameters (Figure 3.4).

[1]http://www.mpbio.com/index.php?cPath=2_77_425

Two different types of FastPrep™ Lysing Matrix tubes were used, i.e. type A and E, which differ in composition of beads. According to the manufacturers homepage[1], the Lysing Matrix A is not designed to be used with soil, however, the Lysing Matrix E is. Due to historical reasons the Lysing Matrix A was used for previous studies at the Department of Microbial Ecology. Unsurprisingly, switching to the Lysing Matrix E brought considerable improvements in extracting RNA from soil (Figure 3.5). Bürgmann *et al.* (2001) reported that bead beating in tubes with comparable composition of beads as the Lysing Matrix E tubes resulted in the highest DNA yield compared to four other types (one of those was similar to the Lysing Matrix A tubes). However, RNA yield is not always linked to DNA yield (as shown in Figure 3.3). Additionally, since the Lysing Matrix A was modified (removal of ceramic sphere), it can not be compared directly with previously reported results, where this modification was not done. Nucleic acids extraction could be improved by grinding the sample in liquid nitrogen in combination with bead beating in Lysing Matrix A tubes. This indicates, that grinding could partly compensate the lacking soil cell lysis capabilities of type A (Figure 3.5). Combining grinding in liquid nitrogen with beat beating in Lysing Matrix E tubes would not have been necessary, because it resulted in no considerable RNA yield increases, but grinding was the only method to separate already frozen samples into smaller aliquots (without thawing).

In the final protocol, a purification step with the OneStep™ PCR Inhibitor Removal Kit was included, because its positive effect on qPCR inhibition could be empirically shown. When this kit was not applied, qPCR was completely inhibited, while when it was applied no such effect was seen with the same type of sample (Figure 3.7). However, how much loss this kit introduces is not clear due to the contradictory results of the quantification. Untreated soil and soil slurry samples were test-extracted and 27–34 % of DNA was lost through this step, while a 67–92 % increase in RNA yield was seen after purification (Figure 3.6). A worst-case loss of 34 % in DNA yield was acceptable, but the increase in RNA yield of nearly 100 % in slurry samples was puzzling. Since an actual increase in RNA is impossible, a bias was most likely introduced by one of the methods. Humic acids can influence the Quant-iT™ RiboGreen® kit, which may lead to underestimation of RNA yield (Mettel *et al.*, 2010). It may be also possible that the TURBO DNA-free™ Kit is affected by coextracts and less RNA is cross-digested by the DNase if they are removed, or that nucleic acids are pelleted because they form complexes with humic acids (Crecchio & Stotzky, 1998) and are therefore lost in the washing step of this kit.

Comparing the results of the first evaluation step with the TNS-based method to the final CTAB-based extraction protocol, an increase in RNA yield from approximately 20 ng to 200–400 ng could be achieved (~ 1000–2000 % increase). The complete final extraction pipeline is shown in Figure A.1.

### 4.1.2. Separation into DNA and RNA

Agarose gel electrophoresis was always done to control nucleic acids quality and quantity. It is a fast and simple method to assess the degree of genomic DNA fragmentation. It was also used for the detection of rRNA bands, but it has a low sensitivity. Absence and presence of (r)RNAs can not be differentiated at low RNA concentrations. A more sensitive and also quantitative electrophoresis method would be Bio-Rad's Experion System or Agilent Technologies' Bioanalyzer (Fleige & Pfaffl, 2006). Other (less expensive) approaches to assess rRNA integrity are denaturing agarose gel electrophoresis (Fleige & Pfaffl, 2006) or polyacrylamide gel electrophoresis (e.g. Zheng *et al.*, 1996; Alm & Stahl, 2000). Since gel electrophoresis is not sensitive enough to verify the complete removal of DNA from RNA samples (and RNA from DNA samples), control PCRs had to be done. DNA-free RNA samples were simply applied to PCR or qPCR assays without any prior cDNA synthesis step. When using standard PCR followed by gel electrophoresis no bands must be visible. When using qPCR the measured $C_q$ must be higher than or equal to the NTC's $C_q$, otherwise the sample is considered contaminated and the DNA removal step has to be repeated or replaced by another method. But repeat-

ing steps will reduce yields, increase the chance of contamination and has negative timely and monetary effects. Small RNA contaminations in the DNA aliquots do not affect quantification (Quant-iT™ PicoGreen® dsDNA Reagent and Kit manual, Invitrogen) and RNA is not amplified by PCR without reverse transcription, therefore the control by gel electrophoresis was sufficient.

Two acceptable nucleic acids separation methods could be determined in the evaluation: (1) AllPrep DNA/RNA Mini Kit without on-column DNase treatment and (2) DNA digestion of an aliquot of the extract. Combination of DNase digestion with another separation method was also tested, because direct digestion cannot be done with the complete samples (one aliquot is needed for its DNA and one for its RNA content). Separation of the sample followed by removal of unwanted DNA/RNA traces by nucleases (if necessary) would circumvent this problem. But apparently the combination of those methods results in higher RNA losses than direct DNase treatment of only half the sample (Figure 3.8). An advantage of the direct digestion method is the possibility to shift yields from DNA to RNA and vice versa. For this analysis, the sample was always separated in two equal aliquots, but one could use a larger portion for one of the nucleic acids. It is also possible to use the complete samples to isolate only DNA or RNA, if the other type is not required. This would automatically double the yield without the need for any other evaluation or improvement steps.

Since the column-based AllPrep DNA/RNA Mini Kit would automatically remove some coextracts from the samples it was selected instead of the direct digestion approach. But as it turned out, the kit introduced foreign DNA to both the RNA and DNA aliquots (Section 3.1.3.2), which was not acceptable. According to the technical support of QIAGEN, the buffers of this kit are not tested for DNA contamination, only for human DNA. It can not be completely excluded that the DNA contamination was introduced by one of the few components not supplied in this kit (e.g. β-mercaptoethanol, ethanol, plasticware), but no supporting evidence was found. So in the final protocol the DNase/RNAse treatment method was used and an additional step of purifying the samples with the OneStep™ PCR Inhibitor Removal Kit was done before the separation. The DNA contamination from the AllPrep DNA/RNA Mini Kit could not be detected when using standard PCR (Figure 3.9), but became obvious by the qPCR approach. The likely reason would be the different sensitivity of PCR product detection with agarose gel electrophoresis (standard PCR) compared to detection by SYBR Green staining (qPCR). Another major difference was the usage of two different recombinant *Taq* DNA polymerases, one by Fermentas used for standard PCR and one by Invitrogen used for qPCR assays (Platinum® *Taq* DNA Polymerase). Since both are proprietary products, no independent comparison of the PCR efficiencies are available and therefore no objective comparison can be done.

### 4.1.3. Reverse transcription of the RNA

The major differences between the two tested kits by Invitrogen, the SuperScript® VILO™ cDNA Synthesis Kit and the SuperScript™ III First-Strand Synthesis System for RT-PCR kit, are their recommended denaturation temperatures of 42 °C and 65 °C, respectively. The SuperScript™ III First-Strand Synthesis System for RT-PCR further includes a RNA removal step by adding and incubating the cDNA with RNase H, and its components are also not premixed. In a previous study comparing different reverse transcriptases the SuperScript III was reported as the most efficient one (Ståhlberg *et al.*, 2004), therefore no other enzymes or kits were tested.

The advertised advantage of the SuperScript® VILO™ cDNA Synthesis Kit is its ability to amplify a wide range in amounts of input RNA with linear results and a decrease in pipetting errors through premixing some solutions. However, denaturing templates with a high G + C content may require higher temperatures than 42 °C, resulting in a loss of detectable diversity with this kit. Microorganisms carrying novel *dsrAB*, that are related to *dsrAB* variants found in Schlöppnerbrunnen, have a G + C

content of about 60 mol% (Mussmann *et al.*, 2005) and the final nucleic acids extraction for qPCR protocol should optimally include those to leave the possibility open for *dsrAB*-based qPCR analysis with the same samples (Steger *et al.*, 2011).

### 4.1.4. 16S rRNA quantification

Since the parameters of the qPCR assays were already evaluated (Pester *et al.*, 2010), the main goal was to test for PCR inhibitors in the slurry samples. In the precursor SIP study (Pester *et al.*, 2010), 5 ng of DNA sample were analysed and tested for PCR inhibition. A DNA dilution series analysed with the 16S rRNA qPCR assay targeting total *Bacteria & Archaea* revealed that even undiluted DNA samples showed no signs of inhibition (0.0049–5.0 µL, which corresponds to 0.059–61 ng were tested, total extract volume was 200 µL; Figure 3.13). This analysis could not be done with the *Desulfosporosinus*-specific 16S rRNA qPCR assay due to the low natural abundance of *Desulfosporosinus* 16S rRNA genes.

RT-qPCR consists of the reverse transcription and the qPCR step, which both depend on enzymes that can be inhibited by different endogenous or exogenous substances (Saleh-Lakha *et al.*, 2011). Current research dictates that constant amounts of RNA should be applied to qPCR assays (Pugniere *et al.*, 2011). Since RNA yields vary between extractions (Saleh-Lakha *et al.*, 2011 and data from this study), two options are available: (1) dilute RNA samples to the same concentrations (but which possibly dilutes inhibitors at different degrees) or (2) add non-target RNA to reach equal concentrations (Pugniere *et al.*, 2011). The method of choice was the first option, for which it was crucial to exclude inhibitory effects within the range of possible dilution factors. Therefore, similar to the inhibition-tests of the DNA-qPCR, RNA dilution series with two test samples were done. Inhibition was only observed when more than 1 µL (= 10.5 or 2.8 ng) of undiluted sample was applied to RT-qPCR (total extract volumes were 200 µL; Figure 3.12).

Another concern was the effect of the reverse transcription reaction mix on the qPCR assay, which contains components that affect DNA hybridisation behaviour, i.e. $K^+$, $Mg^{2+}$, and dithiothreitol (SuperScript™ III First-Strand Synthesis System for RT-PCR manual), since the cDNA is directly applied to the qPCR assay without changing buffers. Linear correlation of applied cDNA volumes and copy numbers was seen when using the *Desulfosporosinus*-specific 16S rRNA primers. But with the total *Bacteria & Archaea* assay, the 16S rRNA copy numbers did not correlate with the applied amounts of cDNA solution (Figure 3.14). The major differences between the *Desulfosporosinus*-specific and total *Bacteria & Archaea* qPCRs (besides primer sequences) are their annealing temperatures (64 °C and 52 °C, respectively) and the binding positions of the primers (603–821 and 1389–1492, *E. coli* numbering, respectively). Problems arising from primer binding positions at the outer parts of the 16S rRNA are discussed below, but since the same cDNA sample was applied to both qPCR assays, only in different dilutions, the source of the problem with the *Bacteria & Archaea* qPCR assay must be related to the reverse transcription-buffer. A possible approach to determine the exact source of this problem would be to model the effect that the reverse transcription-components ($K^+$, $Mg^{2+}$, dithiothreitol) have on the hybridisation behaviour of both primers pairs at their different temperatures (64 °C vs 52 °C), for example by calculating free energy (ΔG) values, but this is (1) beyond the scope of this study and (2) may not be possible because the component-concentrations are not completely disclosed in the Invitrogen manuals. By using the UNAfold two-state melting tool (Markham & Zuker, 2008), ΔG values for the primers pairs with their corresponding annealing temperatures can be calculated (all parameters except temperature, i.e. ionic strength, were left at their default setting and the second sequence was the perfectly matched target). This resulted in ΔG values of −12.6 and −11.8 for DSP603F and DSP821R, respectively (64 °C), and −17.2 and −17.0 to −15.9 for 1389F and 1492R, respectively (52 °C), which indicates only that the primers with the lower annealing temperature form more stable hybrids but offers no explanation for the cDNA dilution series results. Experimental approaches would be to repeat

the cDNA dilution series with constant amounts of reverse transcription reaction mix components, cDNA samples spiked with known amounts of DNA, or by replacing the buffer of the cDNA samples with PCR-grade water.

Further evidence of problems with the 1389F/1492R primers in combination with RT-qPCR was observed in RNA dilution series (Section 3.1.4.1). This analysis showed a *Desulfosporosinus* to *Bacteria & Archaea* 16S rRNA ratio of 7 % in the time point zero sample (untreated soil) and of 1465 % in the microcosm 25 sample (long-term soil slurry). While an increase in 16S rRNAs is logical since growth of *Desulfosporosinus* in the microcosm 25 was already seen in previous analysis (5 % ratio of 16S rRNA genes, Section 3.1.1), a ratio of over 1000 % is unrealistic. One possible explanation could be that the 1389F/1492R primers bind near the end, and the DSP603F/DSP821R primers bind in the middle of the 16S rRNA. If the 16S rRNA is degraded at the 3′ end, e.g. by an 3′-to-5′ exoribonuclease, the total *Bacteria & Archaea* priming site will be removed first and the total *Bacteria & Archaea*, but not the *Desulfosporosinus*, quantification will be affected. Such hybridisation problems of probes with target sites at the 3′ end of the 16S rRNA were reported previously (e.g. Risatti *et al.*, 1994; Kelly *et al.*, 2005). The obvious solution for this problem would be to use (multiple) different broad-range primers with target sites away from the ends of the 16S rRNA (e.g. Caporaso *et al.*, 2011). Another explanation would be that the *Desulfosporosinus* population in Schlöppnerbrunnen has a 16S rRNA sequence which is not recognised by the 1389F/1492R primers and expresses large amounts of rRNA. This theory, however, is not supported by current sequence data. Out of all available, high quality 16S rRNA sequences (SILVA database SSU Ref 106, Pruesse *et al.*, 2007) belonging to the genus *Desulfosporosinus*, 27 are long enough to cover the 1492R priming site (cultured and environmental sequences), and only four of those have a mismatch against the 1492R primer. Additionally, those are weak mismatches (1 × T/G, 3 × G/G; Ludwig *et al.*, 2004) and located at the 5′ end of the primer. But one major bias in this analysis is the fact that not all sequences are long enough to include the 1492R priming site (only 27 out of 99). Based on these findings, it was decided to abandon 16S rRNA quantification of cDNA samples with the qPCR assay targeting total *Bacteria & Archaea*, since the *Desulfosporosinus* 16S rRNA copy numbers could be normalised against the *Desulfosporosinus* 16S rRNA gene copy numbers.

## 4.2. Substrate preferences of sulfate-reducing microorganisms in Schlöppnerbrunnen

To observe biogeochemical processes and natural responses of microorganisms in peatlands in an experimental environment, it is common practice to incubate soil (slurry) micro- and mesocosms and study their responses under different conditions. Such experiments were used to analyse important microbial processes like sulfate reduction (Loy *et al.*, 2004; Knorr & Blodau, 2009; Pester *et al.*, 2010), iron(III) reduction (Küsel *et al.*, 2008; Reiche *et al.*, 2008), nitrification (Palmer *et al.*, 2010), and methanogenesis (Horn *et al.*, 2003; Wüst *et al.*, 2009; Hunger *et al.*, 2011) in the Schlöppnerbrunnen fens.

Since a multitude of microorganisms capable and incapable of dissimilatory sulfate reduction can use the supplemented electron donors (i.e. formate, acetate, lactate, propionate, or butyrate), their turnover in all microcosms is to be expected in such a complex environmental sample. For example, in similar mixed-substrate incubations with and without sulfate amendment, approximately 50 µmol $L^{-1}$ of lactate and 150 µmol $L^{-1}$ of formate were turned over within two days and 100–150 µmol $L^{-1}$ of acetate and propionate were turned over within 4–6 days. In the absence of sulfate, 50 µmol $L^{-1}$ less propionate was turned over than in the presence of sulfate (Pester *et al.*, 2010). In different anoxic incubations, 2 mmol $L^{-1}$ of lactate were turned over to acetate in seven days (Küsel *et al.*, 2008), and 2 mmol $L^{-1}$ of formate were mostly turned over in two days (Wüst *et al.*, 2009), but in incubations amended with 5 mmol $L^{-1}$ of acetate, propionate, or butyrate these were not turned over within 31 days (but methanogenesis was inhibited; Horn *et al.*, 2003). However, if the microbial community is

offered more favourable substrates they may utilise those first. For example, incubations supplemented with monosaccharides resulted in the formation and accumulation of SCFAs (Hamberger *et al.*, 2008). Incubations from this study, where substrates were added (120 µmol $L^{-1}$ every 3–4 days, every 1–3 days in case of formate) at *in situ* concentrations (Schmalenberger *et al.*, 2007; Küsel *et al.*, 2008), showed, with two exceptions, no continuous accumulation of any electron donors. Butyrate-amended microcosm 30, of the long-term incubation experiment, temporarily accumulated acetate in week one and acetate and butyrate accumulated after day 29 as well (Figure B.7f), which may be explained by more favourable endogenous substrates, or a less active or inhibited fermenting/methanogenic community in this replicate. In one short-term incubation microcosm amended with acetate but not with sulfate (number 11), acetate was turned over until day 2–3 but was produced in the following days (Figure B.10e), also indicating that more favourable substrates were present. In the short-term incubations, the substrate turnover was observed at a smaller temporal scale, revealing that all fatty acids were turned over within 1–3 days. In case of acetate and propionate, the turnover was faster then previously observed (Pester *et al.*, 2010). The difference may be explained through a preincubation phase without any supplementation, which was done in the previous, but not in this study.

In the long-term incubations supplemented sulfate was not turned over in any slurry in the first week (Figure 3.15), which coincides with recent literature (Pester *et al.*, 2010). However, in anoxic incubations with sulfate-, but without substrate amendment (Loy *et al.*, 2004), 500 µmol $L^{-1}$ of sulfate were turned over in 6 days (0–10 cm depth) or 17 days (10–30 cm) with no apparent lag phase. In the second week, definite decreases of sulfate concentrations were observed only in slurries with propionate. Only in later weeks turnover of the added sulfate was observed in formate-, lactate- and butyrate-based incubations, but not to the same degree in biological replicates. Especially the replicates with lactate and propionate showed considerable differences in sulfate turnover profiles. No sulfate turnover in acetate-based incubations indicates that the present SRM community could not utilise acetate under the given conditions. A possible explanation for the lag phase in the incubations in this study, but not in previous studies, could be a different seed community of SRMs in the soil used for the slurries, as discussed later in more detail.

In the short-term incubations only slight decreases in sulfate concentrations over the course of the experiment were observed (Figure 3.21). Since substrates were rapidly depleted, a more frequent addition of substrates may have resulted in a stronger decline of sulfate concentrations. The decrease in sulfate concentrations in the butyrate- and lactate-based microcosms between days 0 and 1 is the strongest indication for sulfate-reducing activity (Figure 3.21f, d). After day 1 nearly no butyrate/lactate was left and no further drops in sulfate concentration were detected. Minimal sulfate turnover was also observed in the controls. Based on the assumption that sulfate reduction was going on, SRMs must have been able to use substrates already present in the soil or fen water as an energy source (as in Loy *et al.*, 2004). Therefore, a statement about substrate preference, based only on the sulfate turnover measurements, can not be taken, since decreases in sulfate concentration could have been fuelled by endogenous substrates and not by the added substrate.

However, in addition to concentration measurements, sulfate reduction rates for the short-term incubations were determined (Section 3.3.2). No correlation of supplemented substrate to sulfate reduction rate was observed in controls (no addition of sulfate) and rates of approximately 0–4 nmol $cm^{-1}$ $day^{-1}$ were measured. Distinct differences in sulfate reduction rates between sulfate-addition and absence of sulfate was observed in the lactate- and propionate-amended incubations (> 14 nmol $cm^{-1}$ $day^{-1}$), and also in one control and one butyrate-amended replicate. The measured rates fell within the range of already reported sulfate reduction rates in Schlöppnerbrunnen (mesocosm incubations without substrate or sulfate supplementation, Knorr & Blodau, 2009; *in situ* measurements, Knorr *et al.*, 2009), where sulfate reduction rates varied strongly between time points and depths.

At the beginning of the incubations, small amounts of sulfate were also detected in microcosms without artificial sulfate amendment (24 and 17 μmol $L^{-1}$ in the long- and short-term incubations, respectively). These were most likely endogenous to the soil or fen water samples, since they were well within the range of previously measured concentrations (10–300 μmol $L^{-1}$; Schmalenberger *et al.*, 2007). A contamination of these microcosms with sulfate is unlikely, since special care was taken to clean bottles used for microcosms, fen water, and substrates. Interestingly, in contrast to sulfate-amended microcosms, complete turnover of this endogenous sulfate was observed in all long-term slurries within 5–26 days (except for controls and acetate-amended slurries). This could indicate, that SRMs with a high affinity for low sulfate concentrations were present (such high affinity was observed in marine sediments, e.g.; Tarpgaard *et al.*, 2011), which were inactive in the sulfate-amended slurries. On the other hand, if these microbes were active in all slurries but could only slowly turn over sulfate, this could have been masked in the sulfate-amended slurries by the variation in sulfate additions (sulfate was added with syringes, which are not exact). Active SRMs in these incubations could also simply be K-strategist undergoing slow, but stable growth. Such r-/K-strategist behaviour depended on electron acceptor concentration has, for example, been shown for nitrate (Kim & Kim, 2006).

It was also observed that in some of these long-term slurries with endogenous sulfate, the sulfate level dropped below the detection limit, but was detected again at later time points (for example in long-term microcosms 16, 23, or 29; Figure B.5d, Figure B.6e, or Figure B.7e, respectively). These sulfate fluctuations could be explained by a proposed internal sulfur cycle, which replenishes sulfate pools (Blodau *et al.*, 2007; Pester *et al.*, 2012). All long-term slurries were opened once or twice a week for soil sampling, and thereby their gas phase was replaced with pure nitrogen gas, meaning any gaseous hydrogen sulfide was removed. At some point, this repeated removal of hydrogen sulfide would diminish the sulfate replenishment activity to below detection limits. But with relative standard deviations of about 10 % at this detection range (Section 3.3.3), it can not be ruled out that these fluctuations are technical artefacts.

Spatial and temporal fluctuations in sulfate concentrations and sulfate-reducing activity in Schlöppnerbrunnen were observed in the last decade (e.g. Küsel & Alewell, 2004; Küsel *et al.*, 2008; Knorr *et al.*, 2009; Reiche *et al.*, 2009), which makes incubation experiments strongly dependent on the sampling date, location, and depth. This adds another explanation, why sulfate and substrate profiles sometimes differed from previously reported data. The variance in responses of the microbial community within biological replicates may be explained by the extreme heterogeneity and diversity of soil (Gans *et al.*, 2005). Another factor could be the concept of the rare biosphere (Pedrós-Alió, 2006; Sogin *et al.*, 2006; Pedrós-Alió, 2007), meaning that low-abundant species can, under the right conditions, be very active and become high-abundant, but because of their low abundance they were not equally distributed in all soil slurries. Fleckenstein *et al.* (2011) calculated models that showed that sulfate pools and therefore sulfate reduction is limited to small "hot spots" in the Schlöppnerbrunnen soil matrix (dependent on the hummock structure and water flow). Soil and fen water slurries were mixed in the incubation bottles and no homogenisation step was done beforehand, the reason being to keep the incubations as close to the natural system as possible. Premixing soil and water in a "master bottle" would possibly decrease soil heterogeneity, but making the incubation one step more artificial.

Cultivated *Desulfosporosinus* sp. utilise formate, lactate, and butyrate as electron donors, but not acetate and propionate (Ramamoorthy *et al.*, 2006; Spring & Rosenzweig, 2006; Lee *et al.*, 2009; Vatsurina *et al.*, 2008; Alazard *et al.*, 2010), which means that part of the sulfate turnover in the lactate- and butyrate-based incubations could have been caused by a *Desulfosporosinus* population (as supported by qPCR results in case of butyrate). Sulfate turnover seen in propionate-based incubations was most likely caused by other SRMs, since all described members of the genus *Desulfosporosinus* could not utilise propionate under the conditions tested. The analysis of all microcosms with qPCR could have

given further insights into which part *Desulfosporosinus* played in these incubations, but could not have been done due to time constrains. If it would turn out that *Desulfosporosinus* was responsible for sulfate reduction in the propionate-amended microcosms, this could be explained by either a novel *Desulfosporosinus* species or the possibility that one of the known members can actually utilise propionate, but the appropriate conditions were never provided.

Out of all five substrates, the highest sulfate turnover was observed in butyrate slurries (long-term incubation experiment; Figure 3.15), therefore microcosm 25 and a control (number 29) were chosen to be analysed with qPCR. To capture the short- and long-term responses of the *Desulfosporosinus* population, time points that lay before and after the first indication of net sulfate turnover were chosen. Between days 9 and 16, sulfate turnover was clearly visible in slurries without complete sulfate accumulation (for example Figure 3.15e, f and Figure B.5c). The analysis of time point zero (untreated soil sample) and the earliest incubation sample (day 2) were selected as base points. As a last time point, day 30 was chosen to capture long-term responses.

Based on the substrate turnover profiles of the butyrate incubations, the presence and growth of SRMs was expected. It could be shown that a *Desulfosporosinus* population was indeed growing in one of these slurries. Natural *Desulfosporosinus* abundance of 0.010 % (based on 16S rRNA genes copies compared to total *Bacteria & Archaea* numbers, Figure 3.19e) coincided with a previous SIP-study (Pester *et al.*, 2010). Additionally, in the SIP-study, the *Desulfosporosinus* population grew to 0.2 % after four weeks of incubation, while in the butyrate slurry their abundance reached 0.086 %. This values can of course not be compared directly, since the experimental setups were not the same. The SIP-study supplemented the anoxic incubations with substrate-mixes (without butyrate) after a preincubation phase to deplete all electron acceptors. The sharp increase between time point zero and day 2 could be a technical artefact, since the samples were different. Time point zero was untreated frozen soil and time point day 2 was a soil slurry sample. Differences in nucleic acids yields between those samples (Figure 3.17) indicates as well, that the evaluated protocol was affected by the sample type and origin.

The results from the RT-qPCR were more interesting, since no such data exists from previous studies. The ratio of *Desulfosporosinus* 16S rRNA transcripts to 16S rRNA genes represents cellular rRNA (= ribosomal) content. Increased cellular rRNA content has been linked to increased growth rates in previous studies (e.g. Poulsen *et al.*, 1993; Binder & Liu, 1998) and Muttray *et al.* (2001) could show a linear correlation between 16S rRNA transcripts to gene ratio and growth rate for *Pseudomonas abietaniphila*. However, this relationship is not universal. For example, it has been shown that inhibition of ammonia-oxidizing bacteria had no effect on their cellular rRNA content (Wagner *et al.*, 1995). Starvation of SRM (*Desulfobacter latus*) cells led to an initial decrease in rRNA content, but at some point decreased rRNA levels stayed constant even after continued starvation (Fukui *et al.*, 1996). Under the assumption that cellular rRNA content can be linked to metabolic activity of *Desulfosporosinus* species, the growth of the population in the sulfate-amended microcosm fits very well to their increasing metabolic activity (their increasing 16S rRNA transcripts to gene ratio). In the control without sulfate, however, the same high ratio was observed at an earlier time point, but without growth (Figure 3.19). It should be noted that the analysis shown in Figure 3.19 was not repeated with biological replicates, and therefore it is possible that the observed differences, especially the increase in cellular rRNA content (Figure 3.19c, d), may be outliers or technical artefacts.

The results from the *Desulfosporosinus*-qPCR of the butyrate- and sulfate-amended incubation are supported by current literature. *D. auripigmenti*, *D. lacus*, *D. meridiei*, and *D. orientis*, for example, can all use butyrate as an electron donor in sulfate reduction (Ramamoorthy *et al.*, 2006; Spring & Rosenzweig, 2006). However, *Desulfosporosinus* was also active in the butyrate-based incubation without sulfate supplementation. One explanation would be fermentation of butyrate, but it is not known if

*Desulfosporosinus* species can ferment butyrate, since this was never determined (Rabus *et al.*, 2006; Ramamoorthy *et al.*, 2006; Spring & Rosenzweig, 2006; Vatsurina *et al.*, 2008; Lee *et al.*, 2009; Alazard *et al.*, 2010). Utilisation of the endogenous sulfate pool could also explain the growth and increase in cellular rRNA content of the *Desulfosporosinus* population in the control microcosm (number 29). This theory is supported by the fact the this endogenous sulfate was only detectable on the first day and on day 12, but not in between, or afterwards (in the control without substrate supplementation, the endogenous sulfate concentrations did not change; Figure B.2d–f). Alternatively, the *Desulfosporosinus* population could grow syntrophically by releasing reducing equivalents to methanogens, as it is known from *Syntrophobacter* (Wallrabenstein *et al.*, 1994; Wallrabenstein *et al.*, 1995; Harmsen *et al.*, 1998). The starting concentration in the sulfate-amended microcosm is 20 times higher than in the control microcosm, therefore another explanation for the different responses would be that the affinity for sulfate of the endogenous *Desulfosporosinus* populations may be higher at lower sulfate concentrations (under the conditions provided), as seen in a SRM community in marine sediments (Tarpgaard *et al.*, 2011). It was suggested that the SRM *Archaeoglobus fulgidus* has two different pathways for oxidation of lactate, one used at low and one at high sulfate concentrations (Habicht *et al.*, 2005), and the same could be the case for *Desulfosporosinus* with butyrate as an electron donor. It can, however, not be excluded that the observed variance was caused by a different starting *Desulfosporosinus* population (i.e. different species) used in the soil slurries, since no homogenisation was done.

The overall sulfate and substrate turnover data and the sulfate reduction rates suggest that SRMs in Schlöppnerbrunnen respond fastest to the addition of butyrate, followed by propionate, and then formate and lactate. The only signs of ongoing sulfate reduction in acetate-amended slurries were found in the long-term microcosm 11 (Figure B.4e), where the endogenous sulfate was removed by day 8, and in the acetate enrichment culture, which could turn over sulfate (only in the first enrichment step; Figure B.14a).

Two enrichment cultures could be gained from this experiment (based on formate and lactate, Section 3.4). In the early stages of the lactate-based enrichment, *Firmicutes* were detected. However, at the final stages of the enrichment, these cells were mostly overgrown by members of the *Deltaproteobacteria*. Since the probes used for detection of *Firmicutes* did not cover *Desulfosporosinus* (Loy *et al.*, 2002), this enrichment is an unlikely candidate for isolation of *Desulfosporosinus*. The microbes of the enrichment based on formate could not be phylogenetically assigned, but most likely did not belong to the *Deltaproteobacteria* or *Firmicutes*. Since most cultivated SRMs are part of these two groups (Wagner *et al.*, 2005; Muyzer & Stams, 2008), this enrichment may contain a novel SRM.

## 4.3. Outlook

It would be interesting to further investigate the responses of *Desulfosporosinus* populations in the other microcosms. Additionally, analysis of the same soil slurries/nucleic acids extracts with different qPCRs assay could be done easily, for example to look at Schlöppnerbrunnen *dsrAB* OTUs (Steger *et al.*, 2011). These samples would further be suitable to be analysed with next generation sequencing approaches, like metagenomics, metatranscriptomics or high-throughput 16S rRNA (gene) amplicon sequencing.

Based on the gained results, new incubation experiments could be designed to further investigate the Schlöppnerbrunnen sulfate-reducing community. For example, since evidence was found that the endogenous amounts of sulfate were turned over, looking at SRMs behaviour without artificial addition of sulfate or with addition of inhibitors (e.g. molybdate, Biswas *et al.*, 2009) could reveal new insights.

# Chapter 5. Summary

Natural wetlands are our planet's largest source of atmospheric methane, a greenhouse gas twenty-four times more effective in global warming than carbon dioxide. Wetlands also act as massive carbon sinks by absorbing and storing carbon dioxide. This balance of greenhouse gas fluxes makes wetlands, and especially peatlands, important environments in regard to the global climate. Biological production of methane (methanogenesis) is exclusively performed by methanogenic archaea. In peatlands, the activity of methanogens is strongly linked with the activity of sulfate-reducing microorganisms (SRMs), and therefore the availability of sulfate. SRMs compete directly and indirectly with methanogenic archaea over electron donors and can drastically reduce methane emissions from wetlands. Syntrophic relationships between these two groups were reported as well.

The temperate, acidic, minerotrophic, low-sulfate Schlöppnerbrunnen fen system in Bavaria (Germany) is an interesting habitat for studying SRMs, since 53 novel *dsrAB* species-level operational taxonomic units were found in the last decade (*dsrAB* are functional phylogenetic marker-genes for SRMs). It was shown that in anoxic Schlöppnerbrunnen soil incubations a *Desulfosporosinus* population was responsible for a substantial part of the sulfate-reducing activity and that this genus was part of the active "rare biosphere".

It was previously shown that in anoxic incubations *in situ* amounts of sulfate are turned over when a mix of common substrates for SRMS is added (also at *in situ* concentrations). This study shows substrate utilisation profiles with single-substrate incubations. Sulfate was partly turned over in the presence of formate, L-lactate, propionate, or butyrate, but not acetate. The overall response of the sulfate-reducing community was very heterogeneous between different incubation conditions and biological replicates. Compared to controls without supplemented sulfate, increases in sulfate reduction rates were observed in L-lactate-, propionate-, or butyrate-, but not formate- or acetate-supplemented soil slurries (reduction rates were measured in cooperation with the University of Bayreuth, Germany).

Metabolic activity and growth of *Desulfosporosinus* species in Schlöppnerbrunnen soil slurries amended with butyrate and with or without sulfate were monitored by quantifying 16S rRNA transcript and gene copies with (reverse transcription) quantitative real-time PCR. In the presence of sulfate, the *Desulfosporosinus* population grew from 0.010 % to 0.086 % relative 16S rRNA gene abundance and their 16S rRNA transcript to gene ratio increased by a factor of four over 30 days of incubation. In incubations without sulfate, less growth was observed (0.036 % abundance), but the 16S rRNA transcript to gene ratio increased to the same levels as with sulfate addition after 9 days, but then decreased again by half. This indicates the presence of different *Desulfosporosinus* populations or different metabolic responses by the same population.

Extensive evaluation and optimisation of the nucleic acids extraction and purification methodology was done. Purity, integrity, and suitability of the extracted DNA and RNA for reverse transcription and quantitative PCR were tested and it was shown that 5 ng of DNA and 1 ng of RNA can be analysed with minimal bias with this new protocol pipeline. Total RNA yields could be increased ten- to twentyfold compared to the previously established method.

First steps in the cultivation of (possibly novel) SRMs from the Schlöppnerbrunnen fen system were done. Single-substrate enrichments cultures with formate and L-lactate are being continued.

# Chapter 6. Zusammenfassung

Natürliche Feuchtgebiete sind die weltweit größte Quelle von atmosphärischem Methan (ein vierundzwanzig Mal stärkeres Treibhausgas als Kohlendioxid). Zusätzlich absorbieren und speichern sie enorme Mengen an Kohlendioxid. Das macht Feuchtgebiete, vor allem Moore, zu wichtigen Ökosystemen im globalen Kohlenstoffkreislauf und somit im globalen Klima. Biologische Produktion von Methan (Methanogenese) wird ausschließlich von methanogenen Archaeen durchgeführt. In Mooren ist die Aktivität von Methanogenen verbunden mit der Aktivität von Sulfatreduzierenden Mikroorganismen (SRMs) und daher auch mit dem verfügbaren Sulfat. SRMs konkurrieren mit den methanogenen Archaeen direkt und indirekt um Elektronendonoren, was zu starkem Rückgang von Methan-Emissionen in Feuchtgebieten führen kann. Diese beiden Gruppen können aber auch mutualistische Beziehungen ausbilden.

Die gemäßigten, sauren, mineralstoffreichen und Sulfat-armen Niedermoore Schlöppnerbrunnen I und II in Bayern (Deutschland) sind ein interessantes Ökosystem um SRMs zu studieren, den bisher wurden 53 unbekannte Arten[1] von SRMs gefunden. In einer vorherigen Studie wurde gezeigt, dass die Gattung *Desulfosporosinus* maßgeblich an der Desulfurikation in anoxischen Inkubationen mit Schlöppnerbrunnen-Bodenproben beteiligt war und zu der sogenannten aktiven „rare biosphere“ gehört.

Es wurde bereits gezeigt, dass natürliche Mengen Sulfat in anoxischen Inkubationen mit einen Substrat-Gemisch (zugegeben in natürlichen Konzentrationen) umgesetzt wird. Diese vorliegende Studie zeigt Substratverbrauchsmuster mit jeweils nur einem Substrat. Sulfatumsatz geschah mit den Substraten Formiat, L-Lactat, Propionat und Butyrat, jedoch nicht mit Acetat. Insgesamt wurde eine sehr heterogene Reaktion der SRM-Gemeinschaft beobachtet, sowohl zwischen den verschiedenen Substraten, als auch zwischen den biologischen Replikaten. Im Vergleich zu den Kontrollen ohne Sulfatzugabe, waren Desulfurikationsraten höher in Inkubationen welche mit L-Lactat, Propionat oder Butyrat ergänzt wurden, jedoch nicht in Inkubationen welche mit Formiat oder Acetat ergänzt wurden (Desulfurikationsraten wurden in Kooperation mit der Universität Bayreuth, Deutschland, gemessen).

Stoffwechselaktivität und Wachstum von *Desulfosporosinus*-Spezies wurde in den Inkubationen welchen Butyrat und Sulfat zugesetzt wurde beobachtet. Hierzu wurden 16S rRNA Transkript- und Gen-Kopien mit quantitativer Echtzeit-PCR bestimmt. Bei Vorhandensein von Butyrat und Sulfat wuchs die *Desulfosporosinus*-Population von 0.010 % auf 0.086 % (relatives Vorkommen, basierend auf 16S rRNA Genen) und das 16S rRNA Transkript-zu-Gen-Verhältnis stieg um das Vierfache innerhalb der beobachteten 30 Inkubationstage an. In der Kontrolle mit Butyrat- aber ohne Sulfatzugabe wurde geringeres Wachstum beobachtet (0.036 % Vorkommen), das 16S rRNA Transkript-zu-Gen-Verhältnis stieg zwar nach 9 Tagen genau so hoch an wie in der Inkubation mit Sulfat, sank danach aber um die Hälfte. Das weist darauf hin, dass sich entweder unterschiedliche *Desulfosporosinus*-Spezies in Schlöppnerbrunnen befinden, oder die gleiche Spezies ihren Stoffwechsel anpassen kann.

Ausführliche Evaluationen und Optimierungen der Methodik zu Extraktion und Aufreinigung von Nukleinsäuren aus Schlöppnerbrunnen-Bodenproben wurden durchgeführt. Die Reinheit und Integrität der extrahierten DNA und RNA, sowie ihre Eignung für die Analyse mit reverser Transkription und quantitativer PCR, wurden getestet. Dabei hat es sich gezeigt, dass 5 ng von DNA und 1 ng von RNA die optimale Menge sind um mit dem entwickeltem Protokoll analysiert zu werden. Im Vergleich zu dem bereits vorhanden Protokoll konnte die RNA-Ausbeute um das Zehn- bis Zwanzigfache erhöht werden.

[1] Ungefähre Artebene, basierend auf den funktionellen, phylogentischen Markergenen *dsrAB*.

Erste Schritte für die Kultivierung von (noch nicht bekannten) SRMs aus den Schlöppnerbrunnen Mooren wurden durchgeführt. Anreicherungen mit Sulfat und Formiat respektive L-Lactat werden weitergeführt.

# Appendix A. Supplementary Materials and Methods

**Table A.1** Laboratory suppliers used for this study.

| Abbreviation | Full name and headquarters | Website |
|---|---|---|
| Air Liquide | L'Air Liquide S.A.; Paris, France | airliquide.com |
| Analis | Analis s.a./n.v.; Suarlée, Belgium | analis.be |
| Applied Biosystems | Part of Life Technologies | appliedbiosystems.com |
| Avantor | Avantor Performance Materials; Center Valley, PA, USA | avantormaterials.com |
| Beckman Coulter | Beckman Coulter Inc.; Brea, CA, USA | beckmancoulter.com |
| Bio-Rad | Bio-Rad Laboratories, Inc.; Hercules, CA, USA | bio-rad.com |
| Biostep | Biostep GmbH; Jahnsdorf, Germany | biostep.de |
| Biozym | Biozym Scientific GmbH; Hessisch Oldendorf, Germany | biozym.com |
| Braun | B. Braun Melsungen AG; Melsungen, Germany | bbraun.com |
| Camspec | Spectronic Camspec Ltd.; Garforth, United Kingdom | camspec.co.uk |
| Carl Roth | Carl Roth GmbH + Co. KG; Karlsruhe, Germany | carlroth.com |
| Eppendorf | Eppendorf AG; Hamburg, Germany | eppendorf.com |
| Fermentas | Part of Thermo Fisher Scientific | fermentas.com |
| Fisher Scientific | Part of Thermo Fisher Scientific | fishersci.com |
| GE Healthcare | GE Healthcare (General Electric Company); Little Chalfont, United Kingdom | gehealthcare.com |
| Greiner Bio-One | Greiner Bio-One International AG; Kremsmünster, Austria | gbo.com |
| Hettich Lab Technology | Andreas Hettich GmbH & Co. KG; Tuttlingen, Germany | hettichlab.com |
| Honeywell | Honeywell International, Inc.; Morristown, NJ, USA | honeywell.com |
| HTC | HTC Corporation; Taoyuan, Taiwan | htc.com |
| Invitrogen | Part of Life Technologies | invitrogen.com |
| Life Technologies | Life Technologies Corporation; Carlsbad, CA, USA | lifetechnologies.com |
| Memmert | Memmert GmbH + Co. KG; Schwabach, Germany | memmert.com |
| Merck | Merck KGaA; Darmstadt, Germany | merck.com |
| Millipore | Millipore Corporation; Billerica, MA, USA | millipore.com |
| MP Biomedicals | MP Biomedicals LLC; Santa Ana, CA, USA | mpbio.com |
| Ochs | Glasgerätebau Ochs Laborfachhandel e.K.; Bovenden, Germany | labor-ochs.de |
| Ohaus | Ohaus Corporation; Parsippany, NJ, USA | ohaus.com |
| Olympus | Olympus Corporation; Shinjuku, Tokyo, Japan | olympus-global.com |
| Oxoid | Part of Thermo Fisher Scientific | oxoid.com |
| PEQLAB | PEQLAB Biotechnologie GmbH; Erlangen, Germany | peqlab.com |
| Polymicro | Polymicro Technologies; Phoenix, AZ, USA | polymicro.com |
| Promega | Promega Corporation; Fitchburg, WI, USA | promega.com |
| Qbiogene | Part of MP Biomedicals | qbiogene.com |
| QIAGEN | QIAGEN GmbH; Hilden, Germany | qiagen.com |
| Sanyo | SANYO Electric Co., Ltd.; Moriguchi, Osaka, Japan | sanyo.com |
| Sartorius | Sartorius AG; Göttingen, Germany | sartorius.com |
| Schott | Schott AG; Mainz, Germany | schott.com |
| Sigma-Aldrich | Sigma-Aldrich Co. LLC; St. Louis, MO, USA | sigmaaldrich.com |
| Tecan | Tecan Group Ltd.; Männedorf, Switzerland | tecan.com |
| Thermo Fisher Scientific | Thermo Fisher Scientific Inc.; Waltham, MA, USA | thermofisher.com |
| VWR | VWR International, LLC; Radnor, PA, USA | vwr.com |
| Whatman | Part of GE Healthcare | whatman.com |
| WTW | WTW Wissenschaftlich-Technische Werkstätten GmbH; Weilheim, Germany | wtw.de |
| Zeiss | Carl Zeiss AG; Oberkochen, Germany | zeiss.com |
| Zymo Research | Zymo Research Corporation; Irvine, CA, USA | zymoresearch.com |

**Table A.2** The complete eight weeks incubation and sampling schedule for the long-term peat soil incubation experiment. Sampling abbreviations: *CE* liquid samples for anion concentrations determination by capillary electrophoresis; *Soil* soil slurry samples for nucleic acids extraction. Concentration of sodium sulfate stock solution was 48 mmol $L^{-1}$, of sodium formate, sodium acetate, sodium L-lactate, sodium propionate and sodium butyrate stock solutions (SCFAs) was 24 mmol $L^{-1}$. Values given are amounts added (µL) and final concentrations in parentheses (µmol $L^{-1}$, assumed total volume: 60 mL).

| Day | Sampling | Sulfate addition | SCFAs addition | Formate addition |
|---|---|---|---|---|
| 1 | CE | 250 (200) | 300 (120) | 300 (120) |
| 2 | Soil | | | |
| 3 | | | | 300 (120) |
| 4 | | | | |
| 5 | CE | 130 (104) | 300 (120) | 300 (120) |
| 6 | Soil | | | |
| 7 | | | | |
| 8 | CE | 130 (104) | 300 (120) | 300 (120) |
| 9 | Soil | | | |
| 10 | | | | 300 (120) |
| 11 | | | | |
| 12 | CE | | 300 (120) | 300 (120) |
| 13 | Soil | | | |
| 14 | | | | |
| 15 | CE | 130 (104) | 300 (120) | 300 (120) |
| 16 | Soil | | | |
| 17 | | | | 300 (120) |
| 18 | | | | |
| 19 | CE | | 300 (120) | 300 (120) |
| 20 | | | | |
| 21 | Soil | | | |
| 22 | CE | 130 (104) | 300 (120) | 300 (120) |
| 23 | Soil | | | |
| 24 | | | | 300 (120) |
| 25 | | | | |
| 26 | CE | | 300 (120) | 300 (120) |
| 27 | Soil | | | 300 (120) |
| 28 | | | | |
| 29 | CE | 130 (104) | 300 (120) | 300 (120) |
| 30 | Soil | | | |
| 31 | | | | 300 (120) |
| 32 | | | | |
| 33 | CE | 130 (104) | 300 (120) | 300 (120) |
| 34 | | | | 300 (120) |
| 35 | | | | |
| 36 | CE | 130 (104) | 300 (120) | 300 (120) |
| 37 | Soil | | | |
| 38 | | | | 300 (120) |
| 39 | | | | |
| 40 | CE | 130 (104) | 300 (120) | 300 (120) |
| 41 | | | | 300 (120) |
| 42 | | | | |

| Day | Sampling | Sulfate addition | SCFAs addition | Formate addition |
|---|---|---|---|---|
| 43 | CE | 130 (104) | 300 (120) | 300 (120) |
| 44 | Soil | | | |
| 45 | | | | 300 (120) |
| 46 | | | | |
| 47 | CE | 130 (104) | 300 (120) | 300 (120) |
| 48 | | | | 300 (120) |
| 49 | | | | |
| 50 | CE | 130 (104) | 300 (120) | 300 (120) |
| 51 | Soil | | | |
| 52 | | | | |
| 53 | | | | |
| 54 | Soil[a] | | | |
| 55 | | | | |
| 56 | | | | |

[a]Two out of three soil slurry replicates were completely sampled, one was keep for cultivation attempts.

## A.1. TNS-based step-by-step nucleic acids extraction protocol

This protocol is based on Lueders *et al.*, 2004 and Bodrossy *et al.*, 2006.

1. Put sample in FastPrep™ Lysing Matrix tube.
2. Thaw samples on ice.
3. Quickly add 750 µL $NaPO_4$ buffer (Table 2.11) at room temperature.
4. Quickly add 250 µL TNS solution (Table 2.12) at room temperature.
5. Lyse cells by bead beating for 45 s at 6.5 m $s^{-1}$.
6. Centrifuge at full speed for at least 2 min at 4 °C.
7. Mix equal volumes supernatant and phenol/chloroform/isoamyl alcohol in a new tube.
8. Centrifuge at full speed for at least 5 min at 4 °C.
9. Mix two volumes of supernatant with one volume of potassium acetate solution (Table 2.13) in a new tube.
10. Keep on ice for 1 h.
11. Centrifuge at full speed for at least 5 min at 4 °C.
12. Mix equal volumes supernatant and chloroform/isoamyl alcohol in a new tube.
13. Centrifuge at full speed for at least 5 min at 4 °C.
14. Mix one volume of supernatant with two volumes of PEG 6000 solution (Table 2.14) in a new tube.
15. Centrifuge at full speed for at least 30 min at 4 °C.
16. Wash pellet with 500 µL of ice cold 70 % ethanol.
17. Centrifuge at full speed for at least 5 min at 4 °C.
18. Remove ethanol and dry pellet at room temperature for maximal 5 min.
19. Resuspend pellet in 50 µL of DEPC-treated water.

## A.2. CTAB-based step-by-step nucleic acids extraction protocol

This protocol is based on Griffiths *et al.*, 2000 and Leininger *et al.*, 2006.

1. Put sample in Lysing Matrix tube.
2. Thaw samples on ice.
3. Add 500 µL CTAB/$KPO_4$ buffer (Table 2.9) and mix.
4. Add 500 µL phenol/chloroform/isoamyl alcohol and mix.
5. Lyse cells by bead beating for 30 s at 5.5 m $s^{-1}$ (different bead beating times and speeds were tested, see Section 3.1.1).
6. Centrifuge at full speed for at least 10 min at 4 °C.
7. Mix equal volumes supernatant and chloroform/isoamyl alcohol in a new tube.
8. Centrifuge at full speed for at least 5 min at 4 °C.
9. Mix one volume of supernatant with two volumes of PEG 8000 solution (Table 2.10) in a new tube.
10. Add 20 µg of glycogen (glycogen helps facilitate nucleic acids precipitation).
11. Keep on ice for 2 h.
12. Centrifuge at full speed for at least 1 h at 4 °C [On the recommendation of Dr. Tim Urich (Department of Genetics in Ecology) the centrifugation times were increased from 10 and 5 min to 1 h].
13. Wash pellet with 1000 µL of ice cold 70 % ethanol.
14. If necessary, add another 20 µg of glycogen.
15. Keep on ice for 3 min.
16. Centrifuge at full speed for at least 1 h at 4 °C.
17. Remove ethanol and dry pellet at room temperature for maximal 5 min.
18. Resuspend pellet in 200 µL of DEPC-treated water (the extraction volume in the final protocol is 200 µL. Some evaluation steps were done with extracts solved in 50 µL of DEPC-treated water).

## A.3. Liquid nitrogen grinding step-by-step protocol

Samples are stored at −20 °C or on dry ice in between steps.

1. Prepare mortars, pestles and spatulas:
   a. Clean with water.
   b. Clean with 70 % ethanol.
   c. Clean with 96 % ethanol.
   d. Ignite remaining ethanol.
   e. Pre cool at −20 °C (optional).
2. Label and measure weight of new and empty bead beating tubes.
3. Grind one sample after the other:
   a. Cool mortar and pestle with liquid nitrogen.
   b. Fill mortar completely with liquid nitrogen.

c. Pre-cool empty tubes on dry ice.
d. Defrost sample very shortly at room temperature to get it out of the old tube.
e. Grind sample to fine powder (refill liquid nitrogen if necessary).
f. Transfer sample to new bead beating tube.

4. Measure weight of filled beat beating tubes.

## A.4. Step-by-step protocol for the OneStep™ PCR Inhibitor Removal Kit

This protocol is based on the manufacturer's manual. All steps are performed at room temperature.

1. If spin column is dry, add 500 µL of DEPC-treated water and wait 1 min.
2. Shake spin column gently.
3. Snap off base.
4. Remove cap.
5. Insert into supplied collection tube.
6. Centrifuge at exactly 8000 × *g* for 3 min.
7. Empty collection tube into waste.
8. Add 500 µL of DEPC-treated water to spin column.
9. Wait 1 min.
10. Centrifuge at exactly 8000 × *g* for 3 min.
11. Repeats steps 7–10.
12. Insert into fresh collection tube.
13. Add 200 µL of sample.
14. Wait 1 min.
15. Centrifuge at exactly 8000 × *g* for 1 min; a pellet may form after the samples is eluted (= spin column matrix), do not transfer it to new tube.
16. Transfer aliquot to new tube for DNase treatment (will become the RNA aliquot).
17. Transfer another aliquot to new tube for RNase treatment (will become the DNA aliquot).
18. Put samples on ice and store at −20 or −80 °C.

## A.5. Step-by-step protocol for the TURBO DNA-free Kit

This protocol is based on the manufacturer's manual. All steps are performed at room temperature.

1. Add 0.1 volumes of 10× TURBO DNase Buffer.
2. Add 1 µL of TURBO DNase.
3. Mix gently.
4. Spin down.
5. Incubate in thermal cycler at 37 °C for 30 min.

6. Add 0.1 volumes of vortexed DNase Inactivation Reagent.
7. Mix well.
8. Incubate for 5 min (mix occasionally).
9. Centrifuge at 10 000 × *g* for 2 min.
10. Transfer supernatant to new tube.
11. Put samples on ice and store at −20 or −80 °C.

## A.6. Step-by-step protocol for RNase treatment of DNA

All steps are performed at room temperature. Volumes given are designed for 85 µL of nucleic acids solution.

1. Add 10 µL of RNase ONE 10× Reaction Buffer.
2. Add 5 µL of RNase ONE Ribonuclease.
3. Mix gently.
4. Spin down.
5. Incubate in thermal cycler at 37 °C for 30 min; followed by 70 °C for 15 min.
6. Cool on ice.
7. Add 250 µL of absolute ethanol.
8. Add 10 µL of sodium acetate solution (Table 2.16).
9. Add 20 µg of glycogen.
10. Mix gently.
11. Spin down.
12. Place at −80 °C for 30 min.
13. Centrifuge at full speed for at least 20 min at 4 °C.
14. Remove supernatant.
15. Add 500 µL of ice-cold 70 % ethanol.
16. Centrifuge at full speed for at least 5 min at 4 °C.
17. Remove ethanol and dry pellet at room temperature for maximal 5 min.
18. Add 85 µL of DEPC-treated water.
19. Put samples on ice and store at −20 or −80 °C.

## A.7. Final nucleic acids extraction pipeline

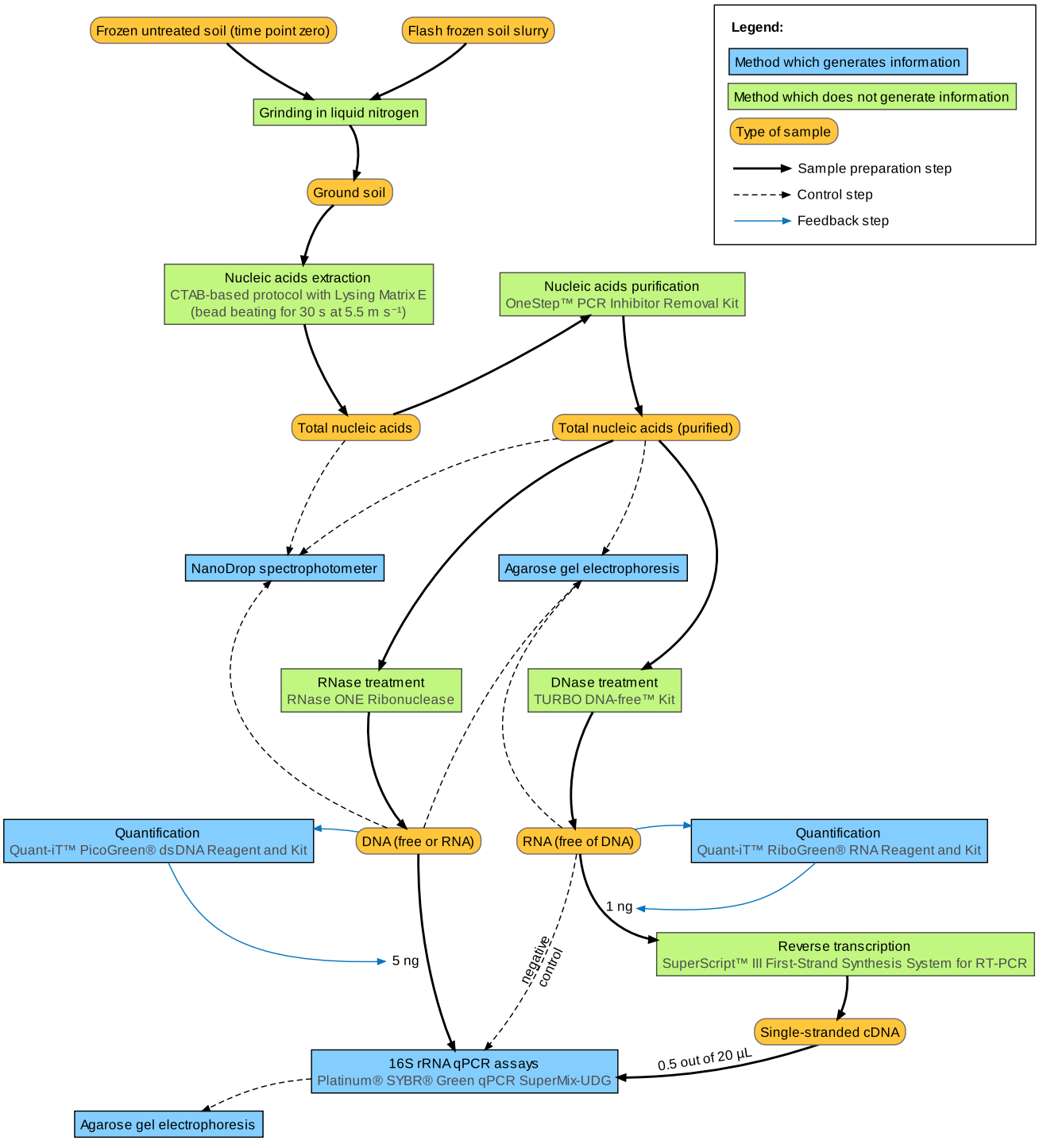


**Figure A.1** The final nucleic acids extraction pipeline from peatland soil (incubation soil slurries) for analysis with qPCR and RT-qPCR.

## A.8. qPCR standards

Table A.3 shows the absolute number of 16S rRNA gene copies in one qPCR reaction with 5 µL of standard DNA dilution. Copy numbers were calculated by using atomic masses of 705705.8 u and 1065411.4 u for one 16S rRNA gene fragment (including flanking vector sequences) of the *Desulfosporosinus* 16S rRNA gene clone SII-2-12 and the *S. wolinii* 16S rRNA gene clone, respectively (Equation A.1). Atomic masses were calculated using the DNA/RNA/Protein/Chemical Molecular Weight Calculator[1] and the sequences listed in Appendix C.

**Equation A.1** Equation to calculate nucleic acids copy numbers in a defined volume from known concentration and atomic mass. *Atomic mass constant* 1 u = $1.660\,538\,921 \times 10^{-27}$ kg (Mohr *et al.*, 2008; Mohr *et al.*, 2011).

$$\text{copy number} = \frac{\text{concentration [ng µL}^{-1}] \times 10^{-12} \times \text{volume [µL]}}{\text{atomic mass [u]} \times \text{atomic mass constant [kg u}^{-1}]}$$

**Table A.3** 16S rRNA gene clone copy numbers in 5 µl of standard solution. Only dilutions $10^{-3}$ to $10^{-9}$ and $10^{-11}$ were used in the total *Bacteria* & *Archaea* and *Desulfosporosinus*-specific qPCR assays, respectively. Values are rounded to a meaningful precession.

| Dilution | *S. wolinii* 16S rRNA gene clone | *Desulfosporosinus* 16S rRNA gene clone SII-2-12 |
|---|---|---|
| $10^{-0}$ | 192 500 000 000 | 380 000 000 000 |
| $10^{-1}$ | 19 250 000 000 | 38 000 000 000 |
| $10^{-2}$ | 1 925 000 000 | 3 800 000 000 |
| $10^{-3}$ | 192 500 000 | 380 000 000 |
| $10^{-4}$ | 19 250 000 | 38 000 000 |
| $10^{-5}$ | 1 925 000 | 3 800 000 |
| $10^{-6}$ | 192 500 | 380 000 |
| $10^{-7}$ | 19 250 | 38 000 |
| $10^{-8}$ | 1 925 | 3 800 |
| $10^{-9}$ | 193 | 380 |
| $10^{-10}$ | 19 | 38 |
| $10^{-11}$ | 2 | 4 |

[1] http://www.changbioscience.com/genetics/mw.html

# Appendix B. Supplementary Results

## B.1. Evaluation of nucleic acids extraction

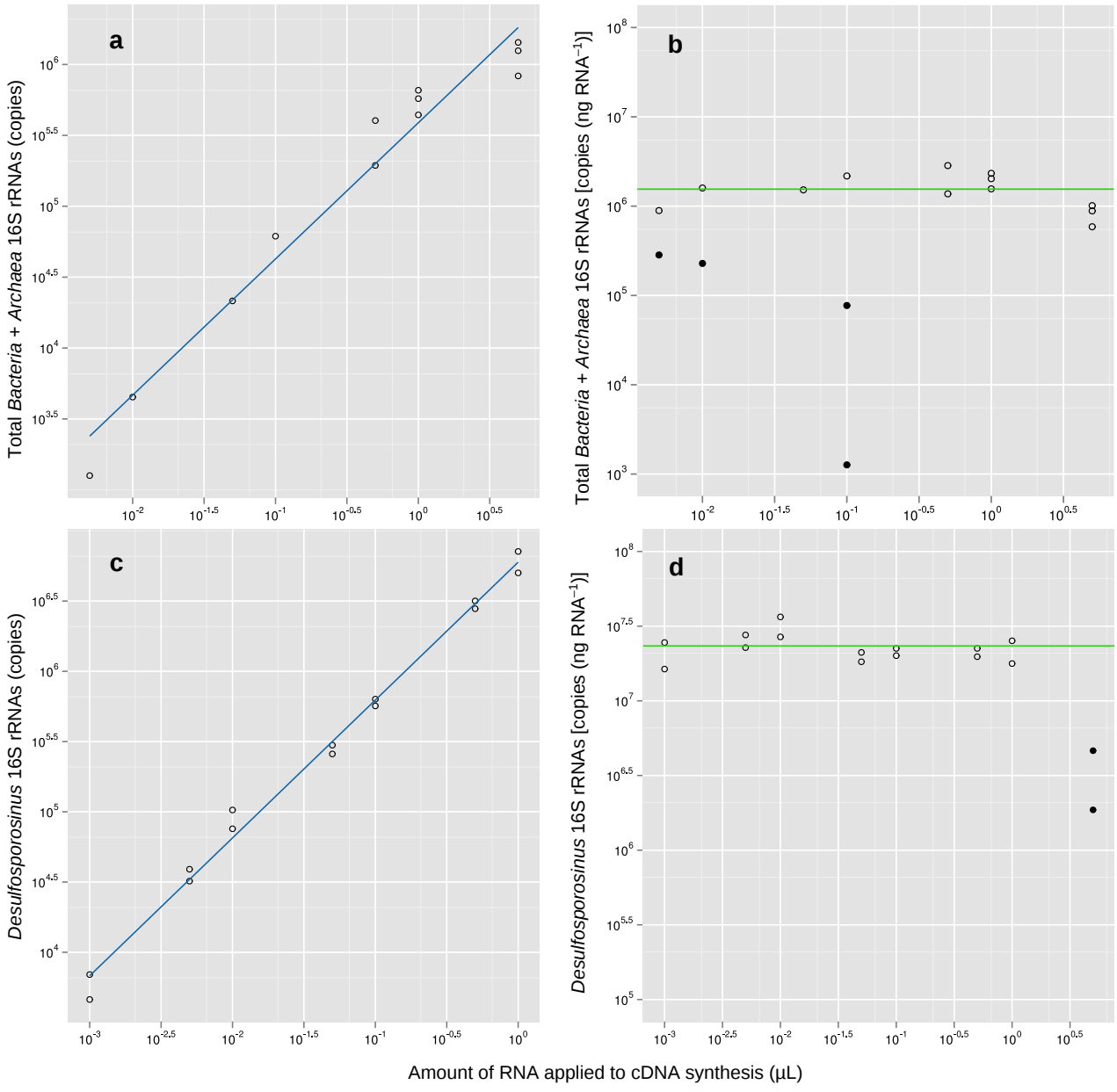


**Figure B.1** Different amounts of purified and DNA-free RNA from microcosm 25 were reverse transcribed (concentration = 2.8 ng µL$^{-1}$). 2 out of 20 µL cDNA solution were then analysed with the 16S rRNA qPCR assays. The data is plotted in absolute copy numbers (plots on the left) and in copy numbers relative to 1 ng of reverse transcribed RNA (plots on the right). Outliers are also shown as filled circles in the right plots. Linear regression analysis of 16S rRNA copy numbers against reverse transcribed volume of RNA extract are shown as blue lines. The horizontal green lines represent the average 16S rRNA gene copy number of this sample (calculated without outliers). **(a)** and **(b)** 16S rRNA qPCR assay targeting total *Bacteria* & *Archaea*. qPCR standard curve quality indicators: $R^2$ = 0.993, PCR efficiency = 92.3 %. Dilution series linear regression quality indicator: $R^2$ = 0.832. Primer 1389Farch was used instead of 1389Fmix in this assay. **(c)** and **(d)** 16S rRNA qPCR assay of the same dilution series targeting *Desulfosporosinus*. qPCR standard curve quality indicators: $R^2$ = 0.999, PCR efficiency = 85.8 %. Dilution series linear regression quality indicator: $R^2$ = 0.965.

## B.2. Peat soil incubations

**Table B.1** All microcosms used in the long-term peat soil incubation experiment. Type of substrate amendment and absence or presence of sulfate amendment given. pH: measured at day 54 (*ND* not determined). Sulfate turnover detected by capillary electrophoresis: (1) for sulfate-amended microcosms: *yes* distinct sulfate turnover; *no* no distinct sulfate turnover; (2) for microcosms without sulfate addition: trace amounts of sulfate from the sample itself were turned over within the experimental timespan (*yes*) or not (*no*), the first day(s) when sulfate was not detected any more are given in parentheses.

| Number | Carbon added | Sulfate added | pH | Sulfate turnover |
|---|---|---|---|---|
| 1 | None | yes | ND | no |
| 2 | None | yes | 4.41 | no |
| 3 | None | yes | 4.22 | no |
| 4 | None | no | 4.30 | no |
| 5 | None | no | 4.25 | no |
| 6 | None | no | ND | no |
| 7 | Sodium acetate | yes | ND | no |
| 8 | Sodium acetate | yes | 4.53 | no |
| 9 | Sodium acetate | yes | 4.49 | no |
| 10 | Sodium acetate | no | 4.55 | no |
| 11 | Sodium acetate | no | 4.65 | yes (8) |
| 12 | Sodium acetate | no | ND | no |
| 13 | Sodium L-lactate | yes | ND | no |
| 14 | Sodium L-lactate | yes | 4.63 | no |
| 15 | Sodium L-lactate | yes | 4.96 | yes |
| 16 | Sodium L-lactate | no | 4.71 | yes (8, 15) |
| 17 | Sodium L-lactate | no | 4.68 | yes (8, 15) |
| 18 | Sodium L-lactate | no | ND | yes (26) |
| 19 | Sodium propionate | yes | ND | yes |
| 20 | Sodium propionate | yes | 4.98 | yes |
| 21 | Sodium propionate | yes | 4.56 | unclear[a] |
| 22 | Sodium propionate | no | 4.68 | yes (22) |
| 23 | Sodium propionate | no | 4.63 | yes (15, 26) |
| 24 | Sodium propionate | no | ND | yes (22) |
| 25 | Sodium butyrate | yes | ND | yes |
| 26 | Sodium butyrate | yes | ND | yes[b] |
| 27 | Sodium butyrate | yes | 4.97 | yes |
| 28 | Sodium butyrate | no | 4.77 | yes (5) |
| 29 | Sodium butyrate | no | ND | yes (5, 15) |
| 30 | Sodium butyrate | no | ND | yes (5) |
| 31 | Sodium formate | yes | ND | yes |
| 32 | Sodium formate | yes | 4.54 | no |
| 33 | Sodium formate | yes | 4.54 | no |
| 34 | Sodium formate | no | 4.98 | yes (22) |
| 35 | Sodium formate | no | 4.92 | yes (12) |
| 36 | Sodium formate | no | ND | yes (22) |

[a]Overall accumulation of sulfate, but time points without sulfate addition showed a decrease in sulfate concentration.
[b]Last sample of this replicate was taken on day 44.

**Table B.2** All microcosms used in the short-term peat soil incubation experiment. Type of substrate amendment and absence or presence of sulfate amendment given. Sulfate turnover detected by capillary electrophoresis: (1) for sulfate-amended microcosms: *yes* distinct sulfate turnover; *no* no distinct sulfate turnover; (2) for microcosms without sulfate addition: trace amounts of sulfate from the sample itself were turned over within the experimental timespan (*yes*) or not (*no*).

| Number | Carbon added | Sulfate added | Sulfate turnover |
|---|---|---|---|
| 1 | None | yes | no |
| 2 | None | yes | no |
| 3 | None | yes | no |
| 4 | None | no | no |
| 5 | None | no | no |
| 6 | None | no | no |
| 7 | Sodium acetate | yes | no |
| 8 | Sodium acetate | yes | no |
| 9 | Sodium acetate | yes | no |
| 10 | Sodium acetate | no | no |
| 11 | Sodium acetate | no | yes |
| 12 | Sodium acetate | no | no |
| 13 | Sodium L-lactate | yes | no |
| 14 | Sodium L-lactate | yes | no |
| 15 | Sodium L-lactate | yes | no |
| 16 | Sodium L-lactate | no | no |
| 17 | Sodium L-lactate | no | no |
| 18 | Sodium L-lactate | no | no |
| 19 | Sodium propionate | yes | no |
| 20 | Sodium propionate | yes | no |
| 21 | Sodium propionate | yes | no |
| 22 | Sodium propionate | no | no |
| 23 | Sodium propionate | no | no |
| 24 | Sodium propionate | no | no |
| 25 | Sodium butyrate | yes | no |
| 26 | Sodium butyrate | yes | no |
| 27 | Sodium butyrate | yes | no |
| 28 | Sodium butyrate | no | no |
| 29 | Sodium butyrate | no | no |
| 30 | Sodium butyrate | no | no |
| 31 | Sodium formate | yes | no |
| 32 | Sodium formate | yes | no |
| 33 | Sodium formate | yes | no |
| 34 | Sodium formate | no | no |
| 35 | Sodium formate | no | no |
| 36 | Sodium formate | no | no |

Sulfate and substrate concentration measurements for each microcosm of the long-term peat soil incubations are plotted in Figure B.2 to Figure B.7 and of the short-term peat soil incubations in Figure B.8 to Figure B.13. Subfigures a–c and d–f are always the slurries with and without sulfate addition, respectively. Black bars indicate amounts of sulfate added. All measurements outside of the standard curve were treated as 0 μmol $L^{-1}$.

The standard curve used to measure the long-term incubations ranged from 12.50 to 1600.00 μmol $L^{-1}$. All peaks were normalised against the internal standard before analysis. Only days 1 to 36 are plotted. Grey dots in sulfate-amended slurries indicate days where no sulfate was added.

Note that in the short-term incubations, the sample for concentration determination was taken before sulfate/substrate addition on day 5. No lines for the sulfate concentration between days 5 and 6 are plotted, because increases were caused by addition of external sulfate, and not by an internal factor. The standard curve used to measure the short-term incubations ranged from 6.25 to 400.00 μmol $L^{-1}$. Normalisation against the internal standard was not necessary.

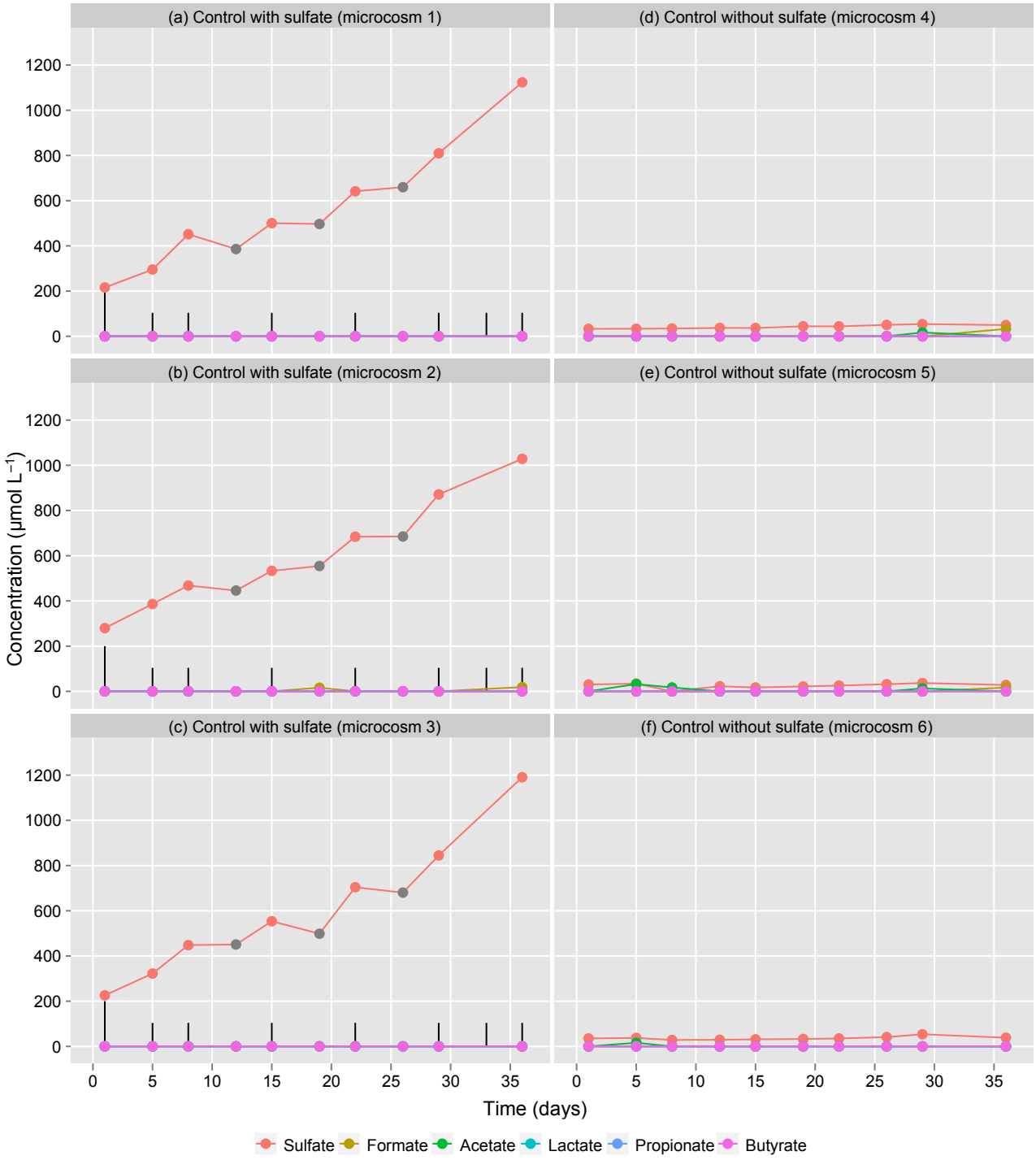


**Figure B.2** Long-term incubation experiment: Control slurries with and without sulfate addition.

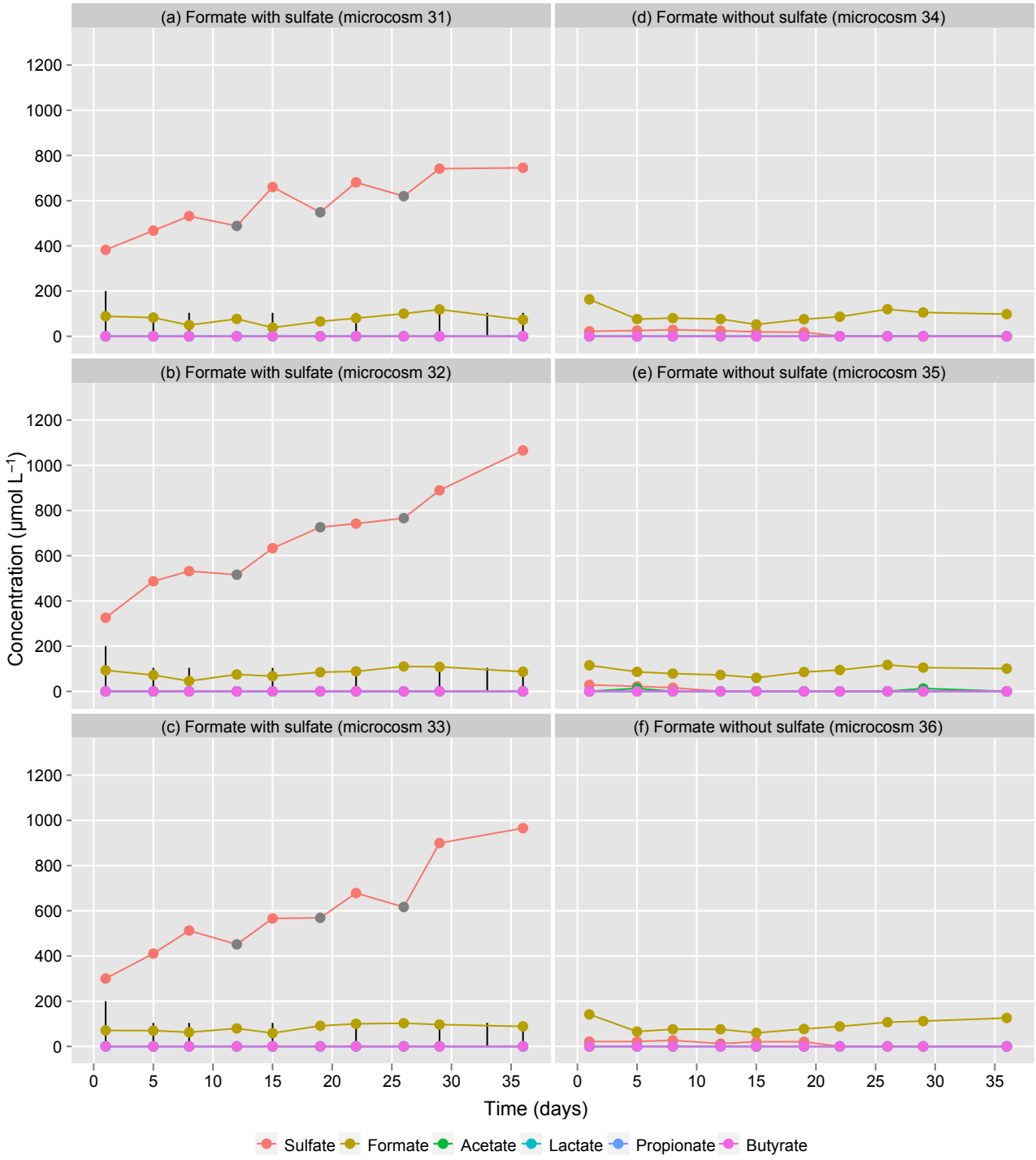


**Figure B.3** Long-term incubation experiment: Formate slurries with and without sulfate addition.

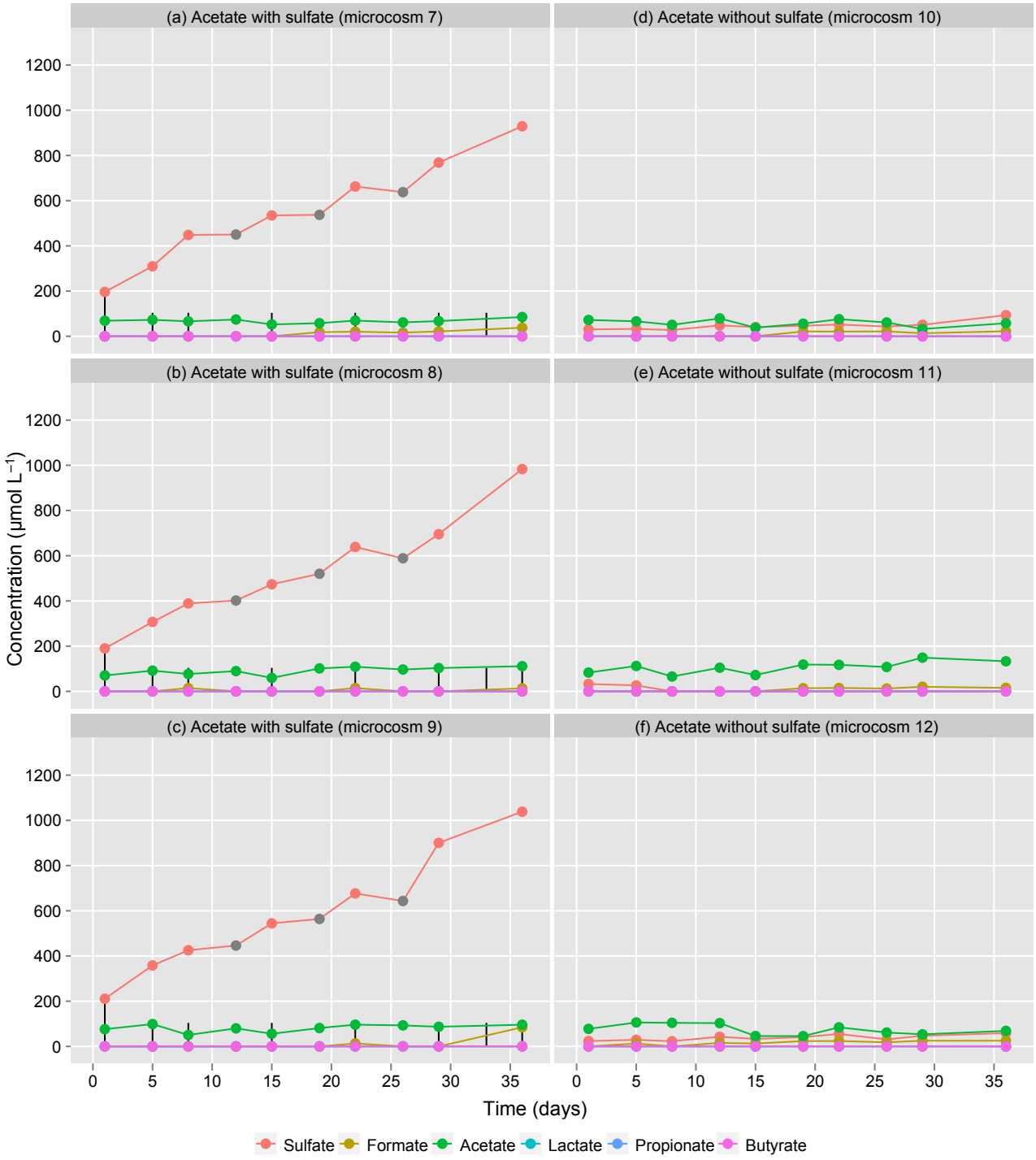


**Figure B.4** Long-term incubation experiment: Acetate slurries with and without sulfate addition.

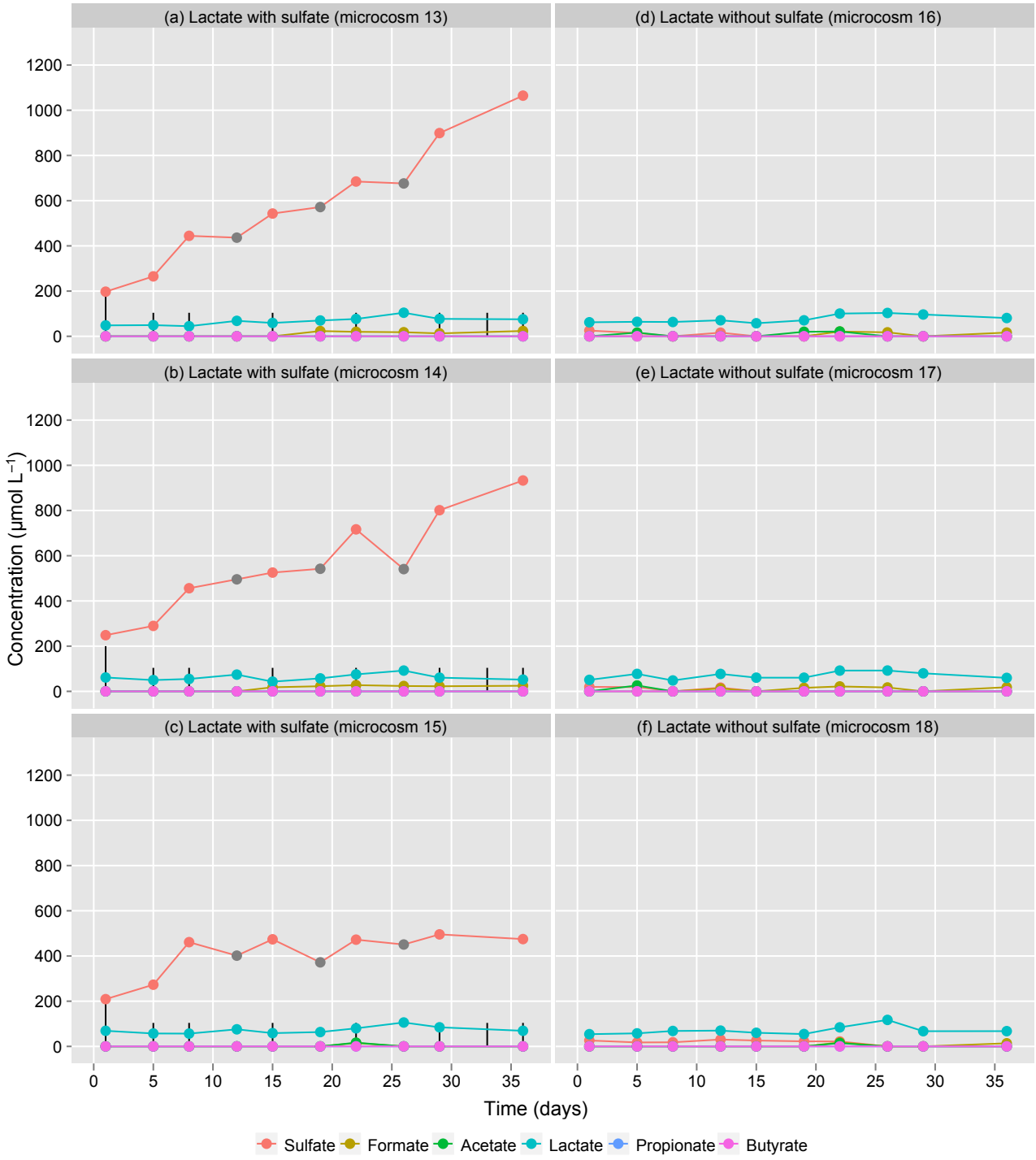


**Figure B.5** Long-term incubation experiment: Lactate slurries with and without sulfate addition.

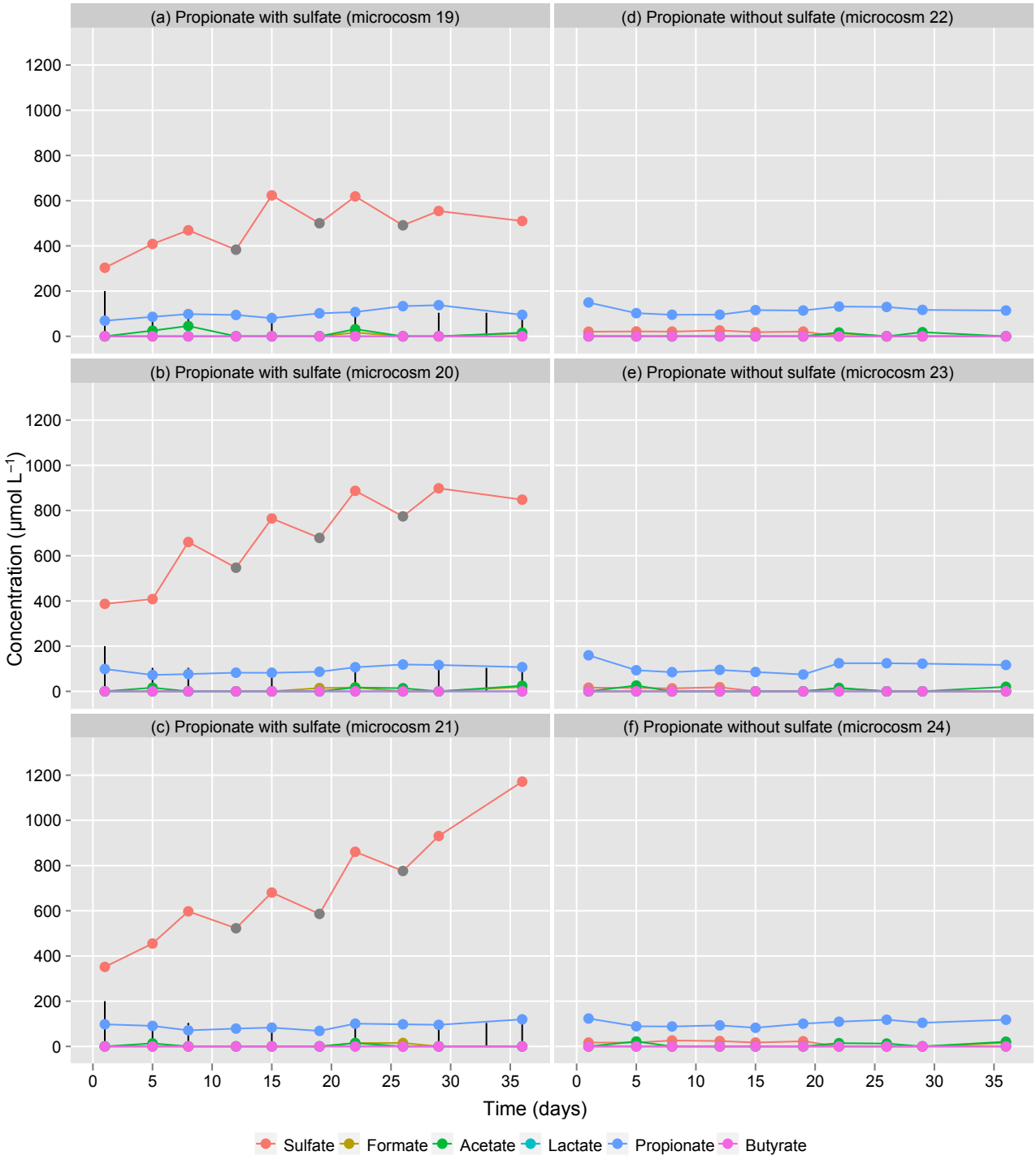


**Figure B.6** Long-term incubation experiment: Propionate slurries with and without sulfate addition.

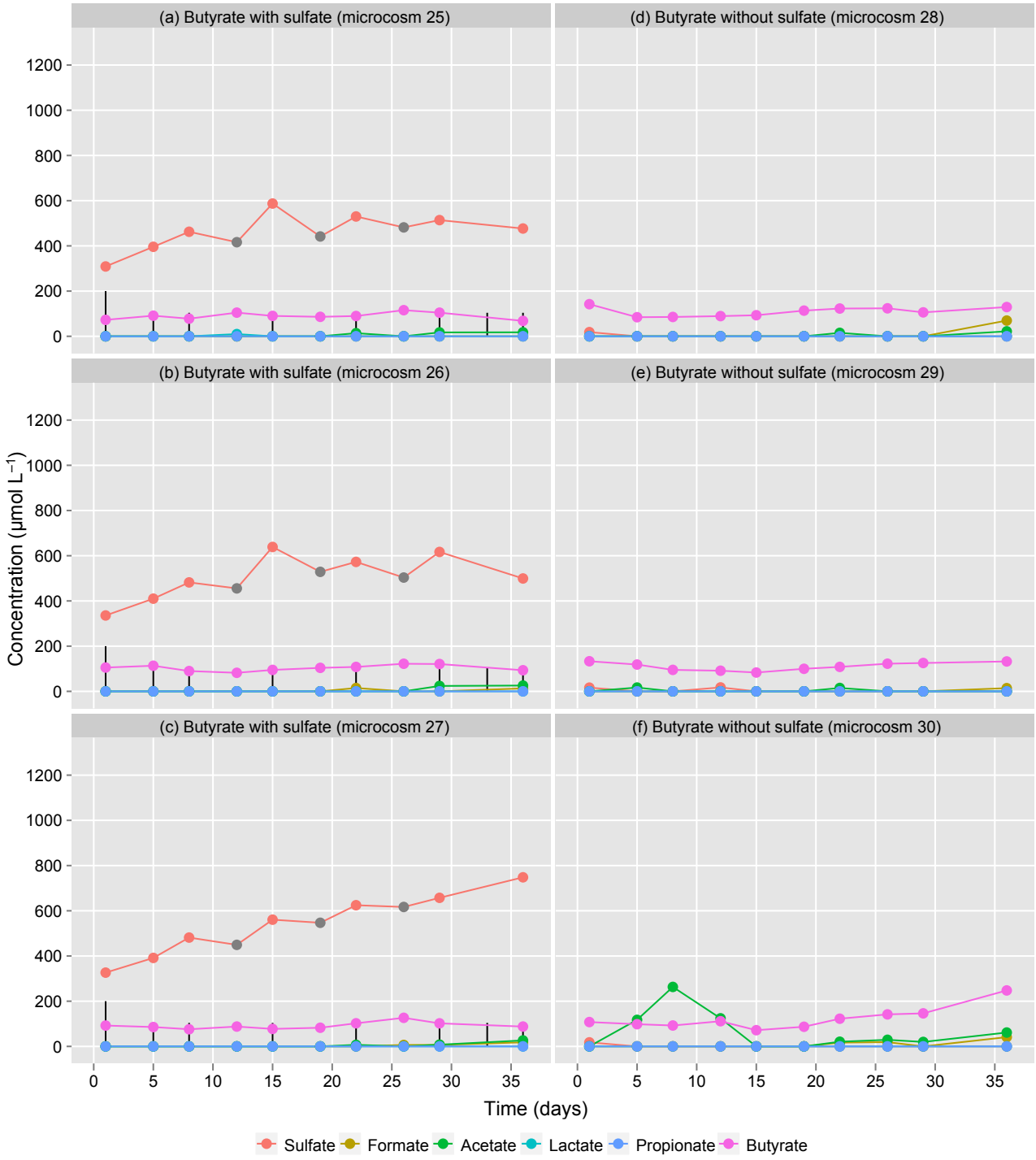


**Figure B.7** Long-term incubation experiment: Butyrate slurries with and without sulfate addition.

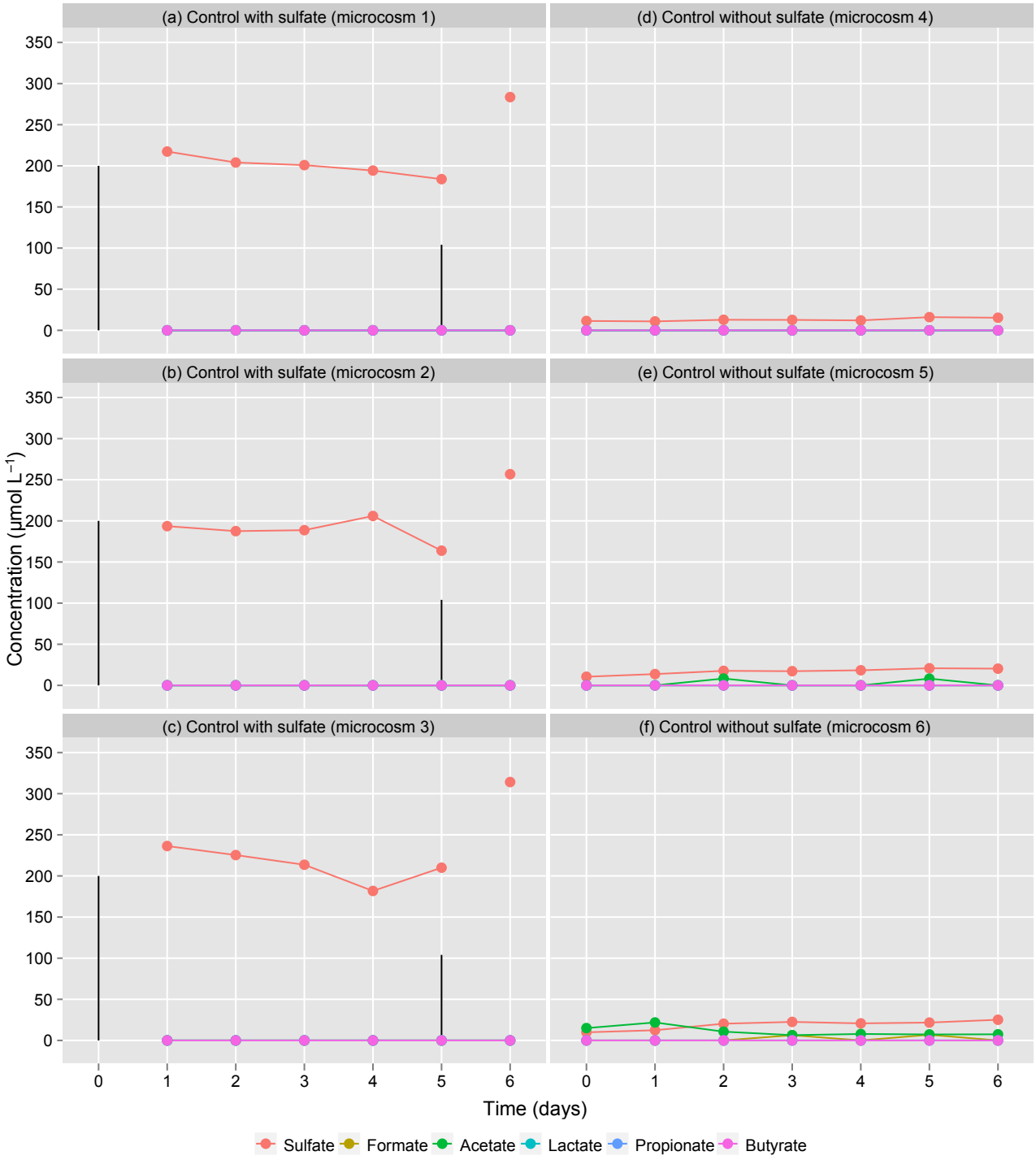


**Figure B.8** Short-term incubation experiment: Control slurries with and without sulfate addition. All sulfate peaks at day 0 in the sulfate-amended microcosms were outliers and are not plotted.

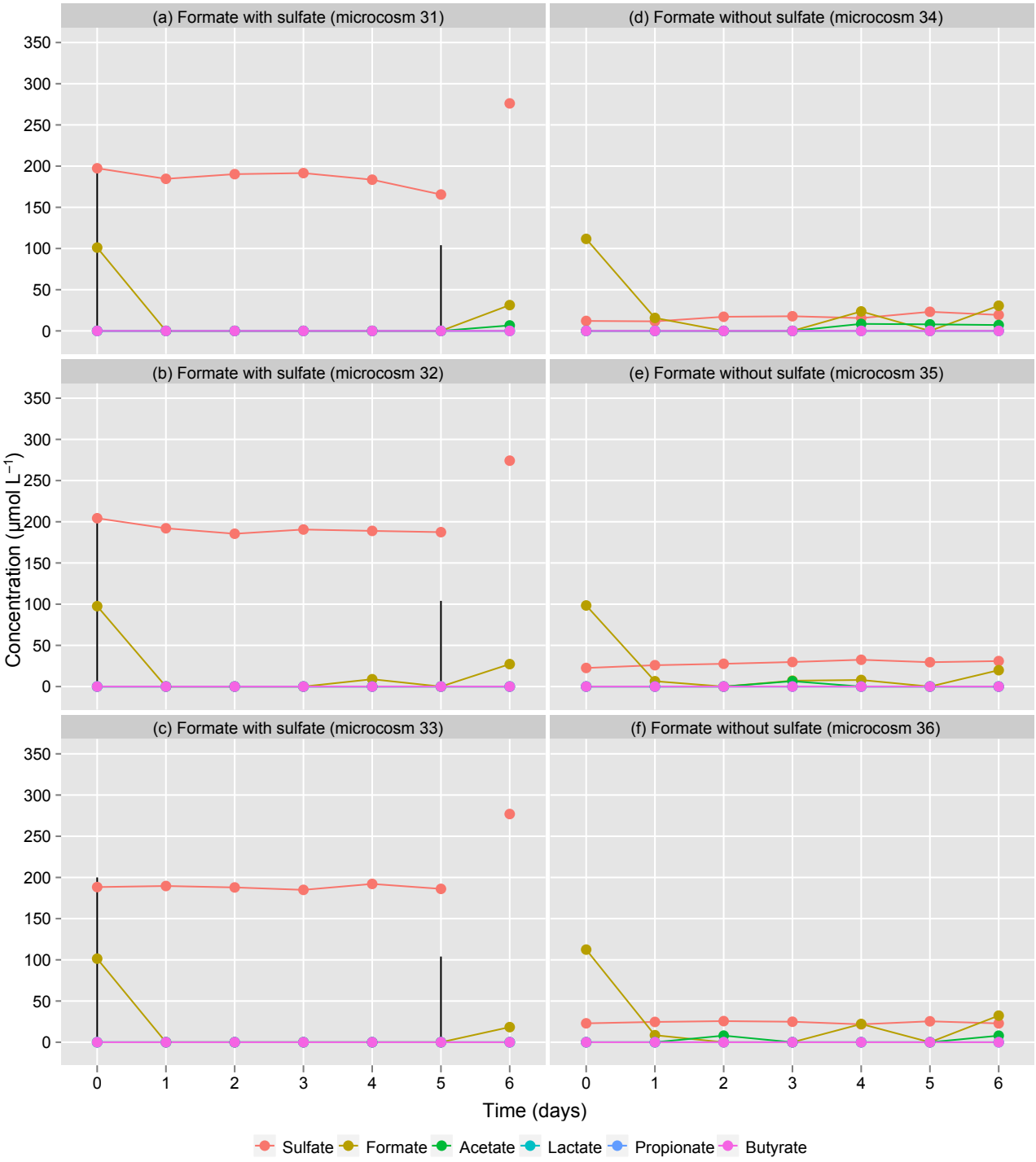


**Figure B.9** Short-term incubation experiment: Formate slurries with and without sulfate addition.

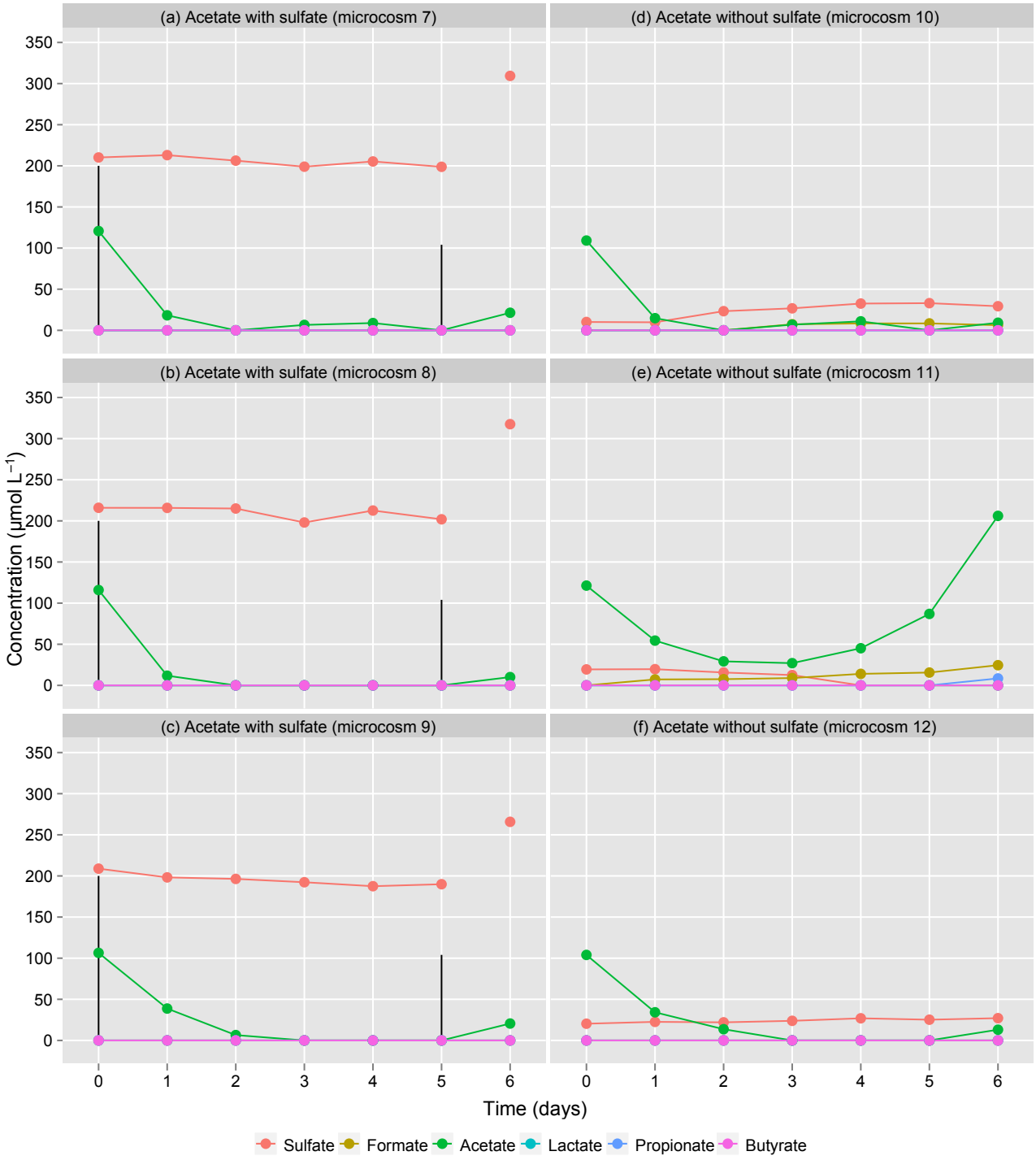


**Figure B.10** Short-term incubation experiment: Acetate slurries with and without sulfate addition.

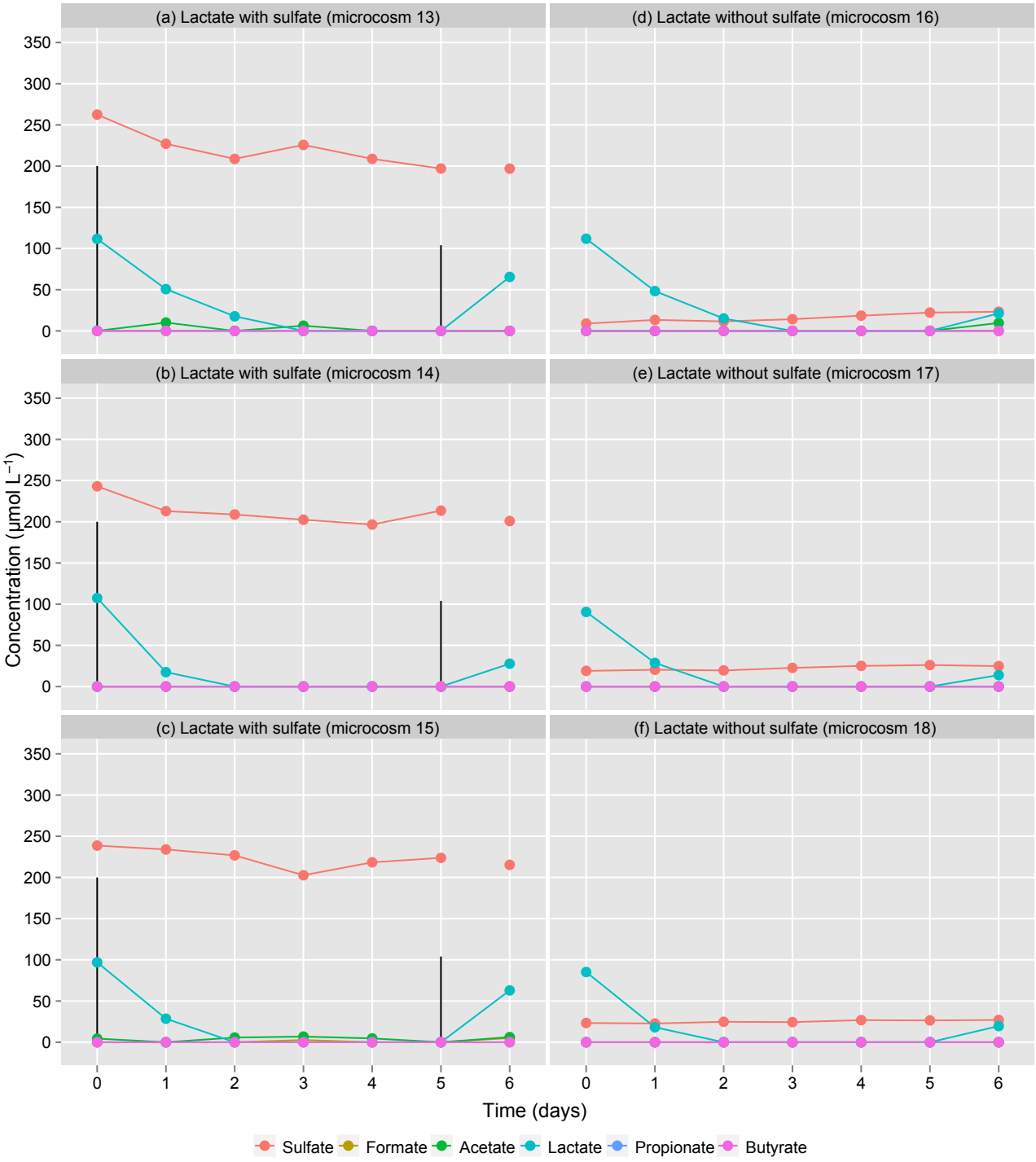


**Figure B.11** Short-term incubation experiment: Lactate slurries with and without sulfate addition.

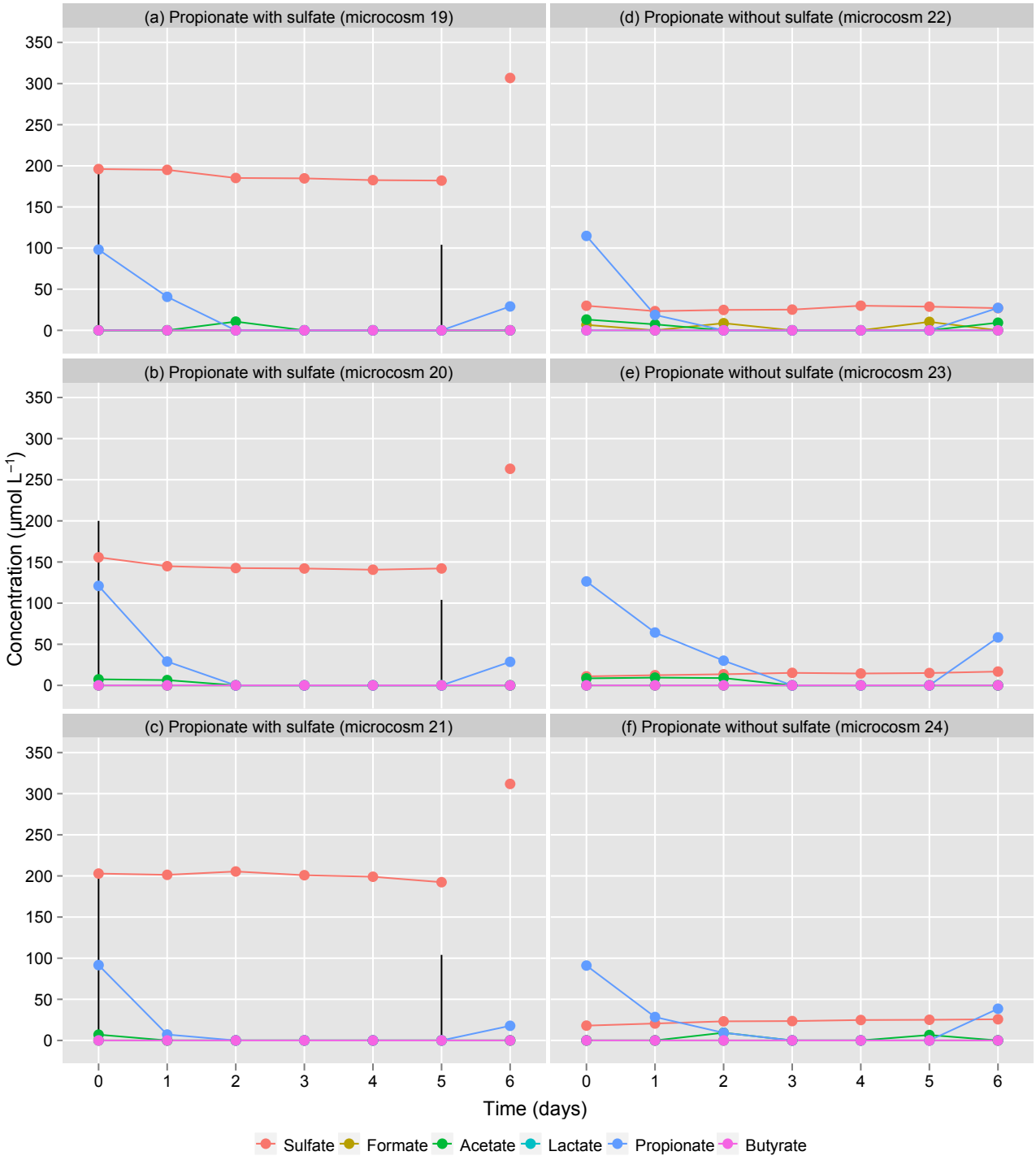


**Figure B.12** Short-term incubation experiment: Propionate slurries with and without sulfate addition.

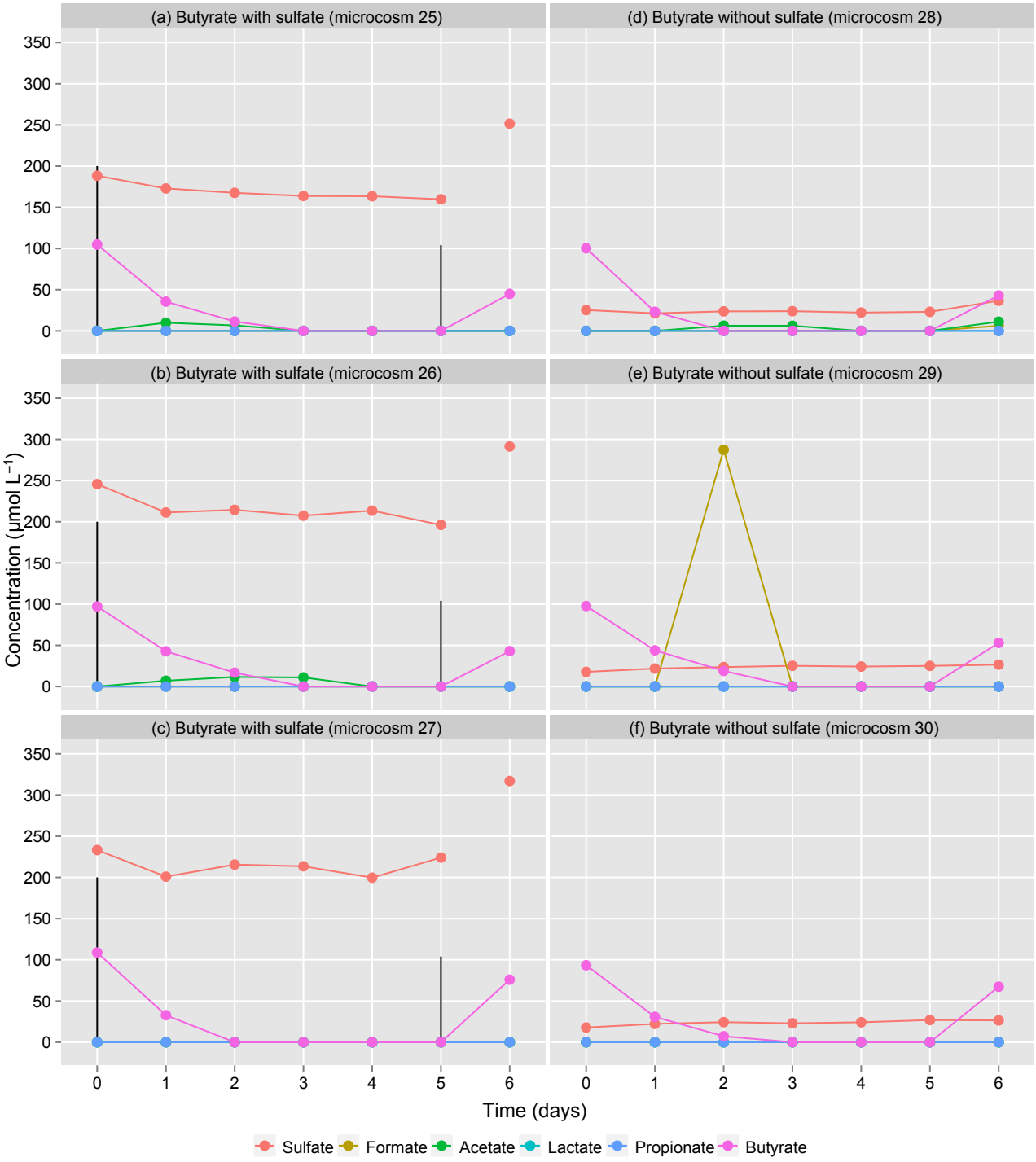


**Figure B.13** Short-term incubation experiment: Butyrate slurries with and without sulfate addition.

## B.3. Enrichment of sulfate-reducing microorganisms

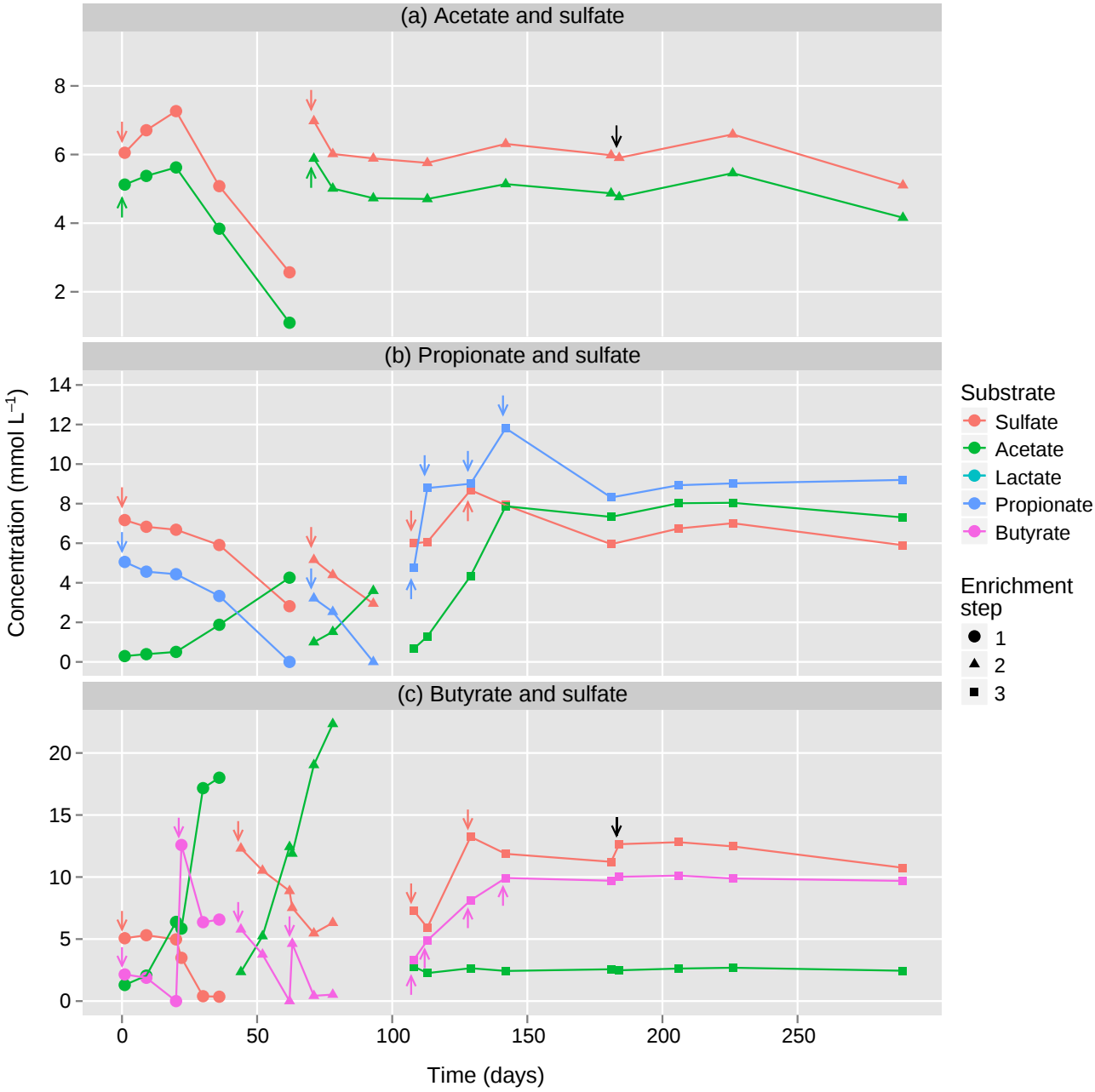


**Figure B.14** Sulfate and substrate turnover profiles in enrichment cultures with sulfate as electron acceptor and acetate (a), propionate (b), or butyrate (c) as electron donors. Inoculations of new tubes (enrichment steps) are indicated by different and not connected symbols. Coloured arrows mark the time points when sulfate/substrates were added (normally one day before samples for capillary electrophoresis measurement were taken). The black arrows mark the addition of yeast extract.

# Appendix C. Sequences

## C.1. *Syntrophobacter wolinii* 16S rRNA gene clone

16S rRNA gene clone sequence of *S. wolinii* (strain DSM 2805) including the flanking sequences of the pCR®2.1-TOPO® vector (from M13 Reverse to Forward primer). Vector and insert sequences are given in 5′-to-3′ direction.

```
CAGGAAACAGCTATGACCATGATTACGCCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGG
AATTCGCCCTTAGAGTTTGATCATGGCTCAGAATAAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAACGAGAAAGG
CTGTTTTCGGACGGCCGAGTAAAGTGGCGSACGGGTGAGTAACRCGTAGATAATCTACCCCTGTGTTCGGGAAACAGTG
CGAAAGCGCTGCTAATACCGGATGCGGCCAGGGGGCGGTGGTTTCCTGGTCAAAGTCGGCCTCTCGCAGAAGCTGATGC
ACGGGGATGAGTCTGCGTCCNATCAGCTAGTTGGTAGGGTAATGGCCTACCAAGGCAACGACGGGTAGCTGGTCTGAGA
GGATGATCAGCCACACTGGCACTGGAACACGNGCCAGACTCCTACGGGAGGCAGCAGTGAGGAATTTTGCGCAATGGCC
GCAAGGCTGACGCAGCAACGCCGCGTGGGTGAAGAAGGCCTTCGGGTCGTAAAGCCCNGTCAGGTGGGAAGAATGTCCC
GGGGAATAATACGCCTCGGGAGTGACGGTACCACCAGAGGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC
GGAGGGTGCGAGCGTTATTCGGAATTACTGGGCGTAAAGCGCGTGCAGGCGGGCGAGCAAGTCTGATGTGAAAGCCCCG
GGCTCAACCTGGGAAGTGCATTGGAAACTGTTCGTCTTGAGTGCTGGAGAGGAAGGGGGAATTCCCGGTGTAGAGGTGA
AATTCGTAGAGATCGGGAGGAATACCAGTGGCGAAGGCGCCCTTCTGGACGGCAACTGACGCTGAGACGCGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGATCACTAGGTGTAGCGGGTACTCATTCCTGC
TGTGCCGCAGCTAACGCGTTAAGTGATCCGCCTGGGGACTACGGTCGCAAGACTAAAACTCAAAGGAATTGACGGGGGC
CCGCACAAGCGGTGGAGTATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCTGGGCTTGACATCCCCGGCCAGGC
ATAGAAATATGTCCTTCCCCTTCGGGGGACCGGGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCCTGCCTTTAGTTGCCTGCGCGTCATGGCGGGCACTCTAGGGGGACTGCCGC
AGTGAATGCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCTTTATGCCCAGGGCTACACACGTACTACAATGGG
CGGTACAAAGGGAAGCGAGCCTGTGAGGGGGAGCCAATCCCAAAAAGCCGTTCACAGTTCGGATCGGAGTCTGCAACTC
GACTCCGTGAAGGTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCG
CCCGTCACACCACGAAAGTCGGCTGTACCAGAAGTGCGTGGGCTAACTCTCAYGAGAGGCAGCGTACCAAGGTATGGTC
GGTAATTGGGGTGAAATCGTAACAAGGTAACCAAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCA
TGCATCTAGAGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTAC
```

Source: Wentrup, 2007

## C.2. *Desulfosporosinus* SII-2-12 16S rRNA gene clone

SII-2-12 16S rRNA gene clone sequence of *Desulfosporosinus* including the flanking sequences of the 16S rRNA amplification primers and the pCR®2.1-TOPO® vector (from M13 Reverse to Forward primer). Vector and insert sequences are given in 5′-to-3′ direction.

```
CAGGAAACAGCTATGACCATGATTACGCCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGG
AATTCGCCCTTAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAACGGAGAATT
TCGAAGAGTTTACTTGGAGAAATTTTTAGTGGCGGACGGGTGAGTAACGCGTGGGTAACCTACCCATAAACCCGGGACA
ACCCTTGGAAACGAGGGCTAATACCGGATAATCTTTGGACTTGGCATCAAGTTTTAAGAAAAGGTGGCCTCTGTATATG
CTACCGATTATGGATGGACCCGCGTCTGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCAGTAGCCGG
CCTGAGAGGGTGAACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATCTTCCGC
AATGGACGAAAGTCTGACGGAGCAACGCCGCGTGTATGATGAAGGTCTTCGGATTGTAAAGTACTGTCTTTGGGGAAGA
ATGATTGGTTTGAAAATATTGAGCCAATATGACGGTACCCAAGGAGGAAGCCCCGGCTAACTACGTGCCAACAGCCGCG
GTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCGCGTAGGCGGATGTTTAAGTCCGGTGTGAA
AGATCAGGGCTCAACCCTGAGAGTGCATCGGAAACTGGGCATCTTGAGGACAGGAGAGGAAAGTGGAATTCCTAGTGTA
GCGGTGAAATGCGTAGATATTAGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGACTGTAACTGAGGCTGAGGCGCGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGAGTGCTAGGTGTAGAGGGTATCGA
CCCCTTCTGTGCCGCAGTTAACACAATAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAATGAATTGA
CGGAAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCAATTCGCCCTATA
GTGAGTCGTATTACAATTCACTGGCCGTCGTTTTAC
```

Source: Pester *et al.*, 2010 (GenBank accession number: GU270660.2)

## C.3. pCR2.1-TOPO vector

Flanking sequences of the pCR®2.1-TOPO® vector (from M13 Reverse to Forward primer) without insert. Vector sequence is given in 5′-to-3′ direction.

```
CAGGAAACAGCTATGACCATGATTACGCCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGG
AATTCGCCCTT-Insert-AAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGG
CCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTAC
```

# Appendix D. Chemical formulas

$SO_4^{2-}$
: sulfate anion

$SO_3^{2-}$
: sulfite anion

$Na_2SO_4$
: sodium sulfate

$H_2SO_4$
: sulfuric acid

$SO_2$
: sulfur dioxide

$H_2S$
: hydrogen sulfide

$HS^-$
: hydrogen sulfide anion

$CO_2$
: carbon dioxide

$HCO_3^-$
: bicarbonate anion

$H_2O$
: water

$CH_4$
: methane

$H_2$
: hydrogen

$N_2$
: nitrogen

NaCl
: sodium chloride

LiCl
: lithium chloride

HCl
: hydrochloric acid

NaOH
: sodium hydroxide

$K^+$
: potassium cation

$Mg^{2+}$
: magnesium cation

# Glossary

| | |
|---|---|
| % (v/v) | volume/volume percentage |
| % (w/v) | weight/volume or more exact mass/volume percentage. Calculated by dividing the weight of the solute (in g) trough the volume of the solution (in mL) times 100. |
| % (w/w) | weight/weight or more exact mass/mass percentage |
| 16S rRNA | rRNA from the small ribosomal subunit |
| 23S rRNA | rRNA from the large ribosomal subunit |
| AOM | anaerobic oxidation of methane |
| bp | base pairs (unit of length for doubled-stranded nucleotide chains) |
| cDNA | complementary DNA |
| CE | capillary electrophoresis |
| CI | chloroform/isoamyl alcohol |
| $C_q$ | quantification cycle (qPCR) |
| CTAB | cetrimonium bromide |
| DAPI | 4′,6-Diamidino-2-phenylindole |
| DEPC | diethylpyrocarbonate |
| dsDNA | doubled-stranded DNA |
| *dsrAB* | the genes that encode the α and β subunits of the dissimilatory (bi)sulfite reductase |
| FISH | fluorescence *in situ* hybridization |
| gDNA | genomic DNA |
| GHG | greenhouse gas |
| KAc | potassium acetate solution |
| kb | kilobases (1000 bp = 1 kb) |
| LCFA | long-chain fatty acids |
| nt | nucleotides (unit of length for single-stranded nucleotide chains) |
| NTC | no-template control (PCR, qPCR) |
| OD | optical density |

| | |
|---|---|
| OTU | operational taxonomic unit |
| PCI | phenol/chloroform/isoamyl alcohol |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| PFA | paraformaldehyde |
| qPCR | quantitative real-time PCR |
| rRNA | ribosomal RNA |
| RSD | relative standard deviation (standard deviation divided by arithmetic mean), normally expressed as a percentage |
| RT | reverse transcription |
| RT-PCR | reverse transcription PCR |
| RT-qPCR | reverse transcription qPCR |
| $R^2$ | coefficient of determination |
| SCFA | short-chain fatty acids |
| SD | standard deviation |
| SDS | sodium dodecyl sulfate |
| SIP | stable isotope probing |
| SRM | sulfate-reducing microorganism |
| ssDNA | single-stranded DNA |
| UV | ultraviolet |
| $\Delta G$ | Gibbs energy |
| $\Delta G'$ | $\Delta G$ at natural/*in situ* conditions |
| $\Delta G^0$ | $\Delta G$ at standard conditions |
| $\Delta G^{0\prime}$ | $\Delta G^0$ at more natural standard conditions |

# References


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