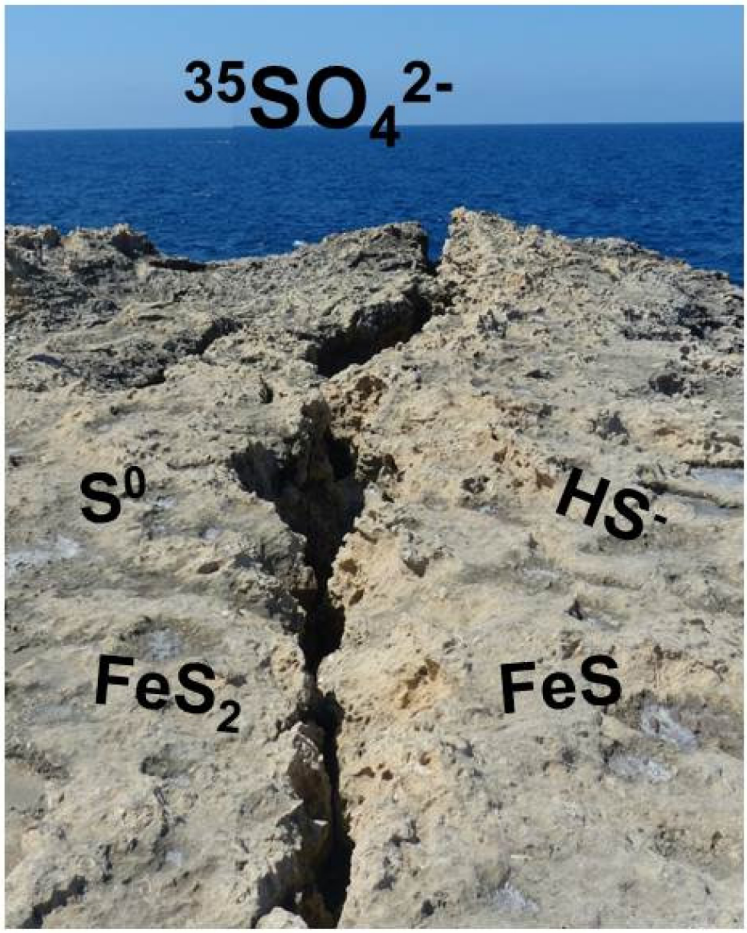

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^{35}S-tracer method for analyzing microbial sulfur compound cycling in oligotrophic anoxic groundwater habitat

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Preface

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Contents

1. Introduction

1.1 Deep groundwater environment – energy challenges for microorganisms

The deep anoxic groundwater with low amount of nutrients (oligotrophic) is a demanding habitat for microorganism. In general microbial cells in deep subsurface aquifers and bedrock fractures catabolize 10^4 to 10^6 fold more slowly than organisms growing in nutrient-rich cultures and survive with energy fluxes that are 1,000-fold lower than the typical culture-based assessments of maintenance requirements (Hoehler and Jørgensen, 2013). The total number of bacteria found in deep groundwater ecosystems may vary by several orders of magnitude between 10^3 and 10^6 cells per mL of groundwater (Griebler and Lueders, 2009; Itävaara et al., 2011; Nyyssönen et al., 2014).

In deep subsurface the energy sources are few, consisting of scarce organic matter and reduced inorganic compounds such as H_2, Mn(II), Fe(II), ammonia and reduced sulfur compounds that can be oxidized to release energy (Table 1). The occurrence of anaerobic terminal electron accepting processes, govern the biogeochemistry and microbiology of subsurface anoxic environments (Heimann et al., 2010). The dominating microbial groups in different deep subsurface aquifers therefore vary based on prevailing lithology and hydrogeochemistry (Nyyssönen et al., 2014; Bomberg et al., 2015b). In the deep subsurface of the Fennoscandian Shield taxonomically and functionally diverse microbial groups have been detected (Itävaara et al., 2011; Nyyssönen et al., 2014; Bomberg et al., 2015a; Sohlberg et al., 2015; Miettinen et al., 2015; Wu et al., 2015). The most common terminal electron acceptors in this anoxic environment are nitrate, Mn(IV), Fe(III), sulfate, and CO_2 in the order of the decreasing energy yields. The interplay of various redox transformations with transport processes gives rise to the characteristic vertical redox stratification observed also in aquatic sediments, with conditions becoming progressively more reducing with increasing depth below the sediment surface.

Table 1. Common electron acceptors and donors for anaerobic subsurface microbiological communities.

	Nitrogen	**Metals**	**Sulfur**	**Carbon**	**Hydrogen**
Electron acceptors	Nitrate Nitrite	Mn(IV) Ferric iron (III)	Sulfate Sulfite Thiosulfate Sulfur S^0	CO_2/HCO_3^-	
Electron donors	Ammonium	Mn(II) Ferrous iron (II)	Thiosulfate Sulfur S^0 Sulfide	Acetate, Formate, Methanol, Ethanol, Methane, Lactate, Humic acids etc.	Hydrogen

The concern of different sulfur compounds, especially sulfide in the deep bedrock aquifers is connected to safety and risk assessment of the final disposal of high radioactive nuclear waste. Sulfate reducing bacteria are a significant group of microorganisms in anaerobic environments. These sulfate reducing bacteria are able to produce sulfide which may cause corrosion of the copper used in the radioactive waste storage capsules. For this reason the measurement, evaluation and understanding of *in situ* formation of sulfide in deep anoxic oligotrophic groundwater environment is outmost important. However, this is challenging due to multiple changing variables in terms of hydrogeochemistry and microbiology in deep subsurface.

2. Microbial metabolism connected with dominant electron acceptors and donors in deep subsurface

Generally microorganisms using the electron acceptor with the highest Gibbs free energy yields dominate over other microbial groups using energetically less favorable electron acceptors (e.g., Lovley and Goodwin, 1988; Hoehler et al., 1998). Higher energy yields support faster growth rates and result in outcompetion of microbial groups using less beneficial electron acceptors (Cord-Ruwisch et al., 1988). For this reason the sulfate reduction in deep subsurface aquifers is strongly dependent on electron acceptors and donors available for microorganisms. Moreover, signs of sulfide formation and active sulfate reduction have been found in deep subsurface aquifers of Fennoscandian Shield (Bomberg et al., 2015b; Miettinen et al., 2015; Wu et al., 2015). On the other hand microbial activity can greatly influence the geochemistry of deep subsurface environments (Lovley and Chapelle, 1995). Common terminal electron acceptors in microbial metabolism in anoxic environment, nitrate, Mn(IV), Fe(III) and sulfate are presented in the following paragraphs.

2.1 Nitrogen metabolism

Oxidized nitrogen compounds nitrate and nitrite are used as electron acceptors for energy production in deep subsurface. This ability has been found in microorganisms belonging to numerous groups of bacteria and archaea. In prokaryotes and in a few filamentous fungi, the reduction of nitrate is a respiratory process. Reduction of nitrate is coupled to ATP synthesis via electron transport chain. The nitrate reduction is catalyzed by the metalloenzyme nitrate reductase (Nar) under anaerobic conditions. Bacteria can express two types of dissimilatory nitrate reductase, which differ in their location: a membrane-bound (Nar) and a periplasmic-bound (Nap) nitrate reductase. Nitrite reduction may proceed also via two nitrite reductases that are evolutionary unrelated but functionally equivalent. The *nir*K gene and the *nir*S gene encode these reductases (Philippot, 2002).

Dissimilatory nitrate reduction to ammonium (DNRA) occurs when nitrate in comparison to organic carbon is limiting (Cole and Brown, 1980). In DNRA nitrate and nitrite are reduced to ammonium and it is assumed to mostly being catalyzed by the periplasmic nitrate reductase complex Nap (Kraft et al., 2011). The ability to carry out DNRA is phylogenetically ubiquitous. DNRA is performed both by chemolithoautotrophic organisms, which use nitrate to oxidize sulfide or other reduced inorganic substrates and by heterotrophic organisms, which use organic carbon as the electron donor. The gene *nrf*A (a nitrite reductase catalyzing conversion of nitrite to ammonia) used as a marker gene for DNRA, has been found in Gamma-, Delta- and Epsilonproteobacteria (Smith et al., 2007) and in members of the Bacteroidetes, Planctomycetes and Firmicutes (Mohan et al., 2004). Many sulfate reducing bacteria are cabable to perform DNRA in the presence of nitrate (Pereira et al., 1996) although the sulfate reduction may be preferred over DNRA if both electron acceptors are present (Marietou et al., 2009). The position of DNRA in comparison to denitrification and the conditions that favor the one or the other nitrate respiration pathway still need to be determined. In addition the dominant organisms carrying out DNRA in aquatic and terrestrial environments remain to be identified (Kraft et al., 2011).

Ammonia oxidation to nitrate is the first step of nitrification and also under anoxic conditions with nitrogen dioxide (Schmidt and Bock, 1997). Ammonia oxidation is performed especially within bacterial phylum Proteobacteria (β- and γ-Proteobacteria), but also by mesophilic Crenarchaea. Nitrite oxidizing bacteria are a phylogenetically diverse functional group. These slow-growing, autotrophic bacteria use ammonia or nitrite oxidation as their sole source of energy. Ammonia oxidation has also been found among heterotrophic bacteria and yeast. However, heterotrophic nitrification consumes energy. Ammonia oxidizing archaea (AOA) have been found in recent years from marine water and soil including suboxic marine waters, sediments, and oxygen-depleted hot-springs (Erguder et al., 2009; Schleper and Nicol, 2010). AOA can also be found over a wide range of pH, temperature, salinity, and phosphate concentrations with some AOA being adapted to sulfidic environments (Erguder et al., 2009; Schleper and Nicol, 2010). AOA are now generally recognized to have the primary control over ammonia oxidation in terrestrial, marine, and geothermal habitats.

Anaerobic ammonium oxidation (ANAMMOX) couples the oxidation of ammonium with the reduction of nitrate, producing nitrogen gas. Several bacteria that are able to perform the anammox pathway have been characterized and affiliated to the order of Planctomycetales (Kraft et al., 2011). Anammox bacteria have a large evolutionary distance among the genera described so far: *Candidatus Brocadia, Kuenenia, Scalindua, Anammoxoglobus* and *Jettenia*. They are typically slow-growing organisms with a doubling time of many days up to several weeks (Strous et al., 1999). Anammox bacteria have been discovered in aquatic habitats that contain oxygen-depleted zones, including marine and freshwater sediments, sea ice, and wastewater treatment plants.

2.2 Sulfur metabolism

Sulfur is one of the most abundant elements on Earth. In rocks and sediments it is mainly present as pyrite (FeS_2) or gypsum ($CaSO_4$) and in seawater it exists as sulfate (Muyzer and Stams, 2008). The sulfur cycle is complex, because sulfur has a broad range of oxidation states, from -2 to +6, from completely reduced to completely oxidized. Sulfur and its compounds may carry out functions of both electron donors and acceptors. In addition sulfur can be transformed both chemically and microbiologically. Furthermore, the sulfur cycle is closely linked to other elemental cycles, such as the nitrogen, carbon and iron cycles.

Sulfur metabolism is profoundly ancient, and genes related to sulfur oxidation and reduction are dispersed throughout the Bacterial and Archaeal domains (Ghosh and Dam, 2009). In the microbial sulfur cycle there is present both assimilative and dissimilative sulfur compound reduction as well as sulfur oxidation and disproportionation reactions. In assimilative sulfate reduction sulfate is taken up as a nutrient and reduced to sulfide, which is then incorporated into sulfur-containing amino acids and enzymes (Böttcher, 2011). Dissimilative sulfur and sulfate reduction reactions generate metabolic energy. In sulfur and sulfate reduction reactions hydrogen sulfide is produced from elemental sulfur or from dissolved sulfate by sulfur- and sulfate- reducing microorganisms (Böttcher, 2011). In addition energy metabolism based on the disproportionation of the inorganic sulfur compounds thiosulfate and sulfite as well as elemental sulfur into sulfide and sulfate has been detected in several sulfate reducing organism (Finster et al., 1998).

In the microbial sulfur cycle, sulfate reduction occurs under strictly anaerobic conditions and uses sulfate as a terminal electron acceptor. Electron donors are usually organic compounds, eventually, hydrogen (Lens, 2009). In the presence of sulfate, sulfate reducing bacteria (SRB) are able to use several compounds as electron donors: formate, acetate, methanol, propionate, butyrate, higher and branched fatty acids, lactate, ethanol, higher alcohols, fumarate, succinate, malate, sugars, carbon monoxide, amino acids and aromatic compounds. Generally, polymeric organic compounds, such as starch, cellulose, proteins, nucleic acids (DNA and RNA) and fats are not direct substrates for SRB (Lens, 2009; Muyzer and Stams, 2008). SRB can establish two different hydrogenogenic life styles termed as sulfidogenic and acetogenic (Plugge et al., 2011). As sulfidogens they reduce sulfate and produce sulfide and as acetogens they ferment puryvate to form acetate, hydrogen and carbon dioxide or they are able to oxidize lactate and ethanol to acetate (Muyzer and Stams, 2008). The advantage of having different metabolic capabilities is that it raises the chance of survival in environments when electron acceptors become depleted (Plugge et al., 2011). Sulfate reducing prokaryotes are a very diverse group of anaerobic bacteria that are widely present in nature and play an imperative role in the global cycling of carbon and sulfur.

The oxidation of reduced sulfur compounds in anoxic conditions by bacteria pro-

ceeds using nitrate as an electron acceptor (Ghosh and Dam, 2009). Several Gammaproteobacteria species as well as some Epsilonproteobacteria species are anaerobic sulfur oxidizing chemolithotrophs. These sulfur oxidizing Epsilonproteobacteria have been detected from the Fennoscandian Shield deep aquifers (Wu et al., 2015; Miettinen et al., 2015; Bomberg and Itävaara, 2012). In addition several sulfur oxidizing chemolithoautotrophic hyperthermophilic archaea can also use an array of electron donors, including H_2S, S, $S_2O_3^{2-}$, $S_4O_6^{2-}$ and sulfide minerals (Amend and Shock, 2001).

2.3 Iron metabolism

Based on mass, iron is the most common element on Earth and fourth most abundant element in Earth's crust. Depending upon the geochemical conditions, iron occurs in nature either in soluble forms or in a variety of minerals (Schwertmann and Fitzpatrick, 1992). In most anoxic ecosystems Fe(III) (ferric iron) minerals are the dominant electron acceptors for bacteria and archaea (Straub, 2011b). At neutral pH Fe(III) oxides are poorly soluble. The solubility of Fe(III) oxides increases with increasing acidity and below pH 2.5 ferric iron is water-soluble (Cornell and Schwertmann, 2003).

Dissolved ferrous iron Fe(II) concentrations are controlled by precipitation-dissolution reactions as well as adsorption processes. Fe(II) precipitates with carbonates, phosphates and sulfides and tends to adsorb to soil particles and minerals (Straub, 2011a). The redox state and bioavailability of Fe(III) and Fe(II) are dynamically controlled by the prevalent redox conditions. The redox potential of the redox pair Fe^{3+}/Fe^{2+} is +770 mV only at acidic pH values. At neutral pH, the redox potential ranges between +100 and -100 mV, while the redox potential of more crystalline Fe(III) oxides (goethite, hematite, magnetite) may be as low as -300 mV (Straub et al., 2001).

The reduction ability of Fe(III), ferric iron, to Fe(II), ferrous iron occurs widely within the domains bacteria and archaea. The electrons for Fe(III) reduction derive mainly from the metabolic oxidation of organic compounds or hydrogen (Straub, 2011b). Most known prokaryotes that grow by Fe(III) reduction belong to the phylum Proteobacteria with representatives in each of the five subdivisions. In addition some bacteria from the Phylum Firmicutes are known to reduce Fe(III) (Lovley et al., 2004). Dissimilatory Fe(III)-reducing prokaryotes receive energy by coupling the oxidation of an electron donor to the reduction of Fe(III). In the absence of Fe(III), prokaryotes either grow by fermentation or utilize alternative electron acceptors like oxygen, nitrate, manganese, reduced sulfur compounds and fumarate. Fermentation end products such as acetate, ethanol, and hydrogen as electron donors are utilized by the majority of Fe(III)-reducing prokaryotes (Straub, 2011b). Fe(III) oxides are poorly soluble at neutral pH and prokaryotes have developed different strategies to transfer electrons from the cell to the surface of Fe(III) minerals.

Hydrous Fe(III) oxides may be reduced by hydrogen sulfide abiotically to elemental sulfur and ferrous sulfide (FeS) species (Yao and Millero, 1996). The reduction rate is strongly dependent on pH with a maximum near 6.5. The divalent ions sulfate, Mg^{2+} and Ca^{2+} decrease the reaction rate. Abiotic reduction of ferric iron Fe(III) and microbial sulfur reduction can in some circumstances be closely connected. Depending on groundwater pH, dissimilatory metal-reducing bacteria can respire alternative electron acceptors to survive, including elemental sulfur (S^0). By combining thermodynamic geochemical modeling with bioreactor experiments using *Shewanella oneidensis* MR-1, Flynn et al. (2014) studied the interplay of Fe/S cycling under alkaline conditions. They demonstrated that under neutral conditions *S. oneidensis* reduced both S^0 and goethite (a-FeOOH) but under alkaline conditions only S^0. This is explained by the thermodynamic models showing that goethite reduction yields as much energy as sulfate reduction at pH ~ 8 but considerably less than S^0 reduction above pH 7. Reduction of S^0, is energetically favorable at any pH and becomes more favorable as pH increases. Even though goethite was not microbiologically reduced at alkaline conditions this occurred abiotically as HS^- produced by microorganisms reduced goethite to Fe^{2+} (Flynn et al., 2014).

Anaerobic dissimilatory Fe(II)-oxidizing prokaryotes utilize Fe(II) as electron donor in anaerobic respiration, i.e., they gain energy by coupling the oxidation of Fe(II) to the reduction of nitrate. Oxidation of Fe(II) to Fe(III), produces only one electron and thus large quantities of Fe(II) need to be oxidized to gain enough energy for maintenance and growth. These oxidation reactions lead to the formation of Fe(III) minerals which are barely soluble at neutral pH (Straub, 2011a). These species, mostly Proteobacteria and Archaea (*Archaeoglobales*), are able to utilize alternative electron donors (e.g., hydrogen, organic acids) and alternative electron acceptors (nitrite, oxygen, ferric iron) (Straub, 2011a). For this reason they are divided into those that are autotrophic, and those that use organic materials as carbon sources and can also grow as heterotrophs (Hedrich et al., 2011). In addition to microbiological oxidation of Fe(II) chemical oxidation reactions may happen and they depend on the pH and appropriate oxidants. In the absence of molecular oxygen, Fe(II) can be chemically oxidized at appreciable rates only by nitrite or Mn(IV) (Straub, 2011a).

2.4 Manganase metabolism

Manganese is the ninth most abundant metal in the Earth's upper continental crust. In the environment, oxidized manganese is found in minerals like birnessite and pyrolusite. Manganese oxides are some of the strongest naturally occurring oxidizing agents in the environment (Tebo et al., 2005). Mn oxides participate in a wide range of redox reactions and they exist in several oxidation states ranging from 0 to +7; however three naturally occurring oxidation states +2, +3, and +4 have biological importance. Only manganese in the oxidation state +2 can occur

as a free ion in aqueous solution. Complexed manganese in the +3 oxidation state can occur in aqueous solution but the free +3 ion has the disposition to disproportionate into the +2 and +4 oxidation states. The +4 oxides of manganese exist mainly as MnO_2 and are insoluble in water (Das et al., 2011). Many dissimilatory metal-reducing bacteria can use Mn oxides as the terminal electron acceptor for the oxidation of organic matter or H_2 in the absence of oxygen.

Large number of microorganisms capable of reduction of Mn(IV) to Mn(II) are known. However, many of these are isolated as iron reducers from environments dominated by iron reduction and are only afterwards shown to reduce manganese oxides. Most properties of iron reducers and iron-reducing communities have been supposed also to apply to manganese reducers (Vandieken et al., 2012). Only few species have been isolated with manganese oxide. Vandieken et al. (2012) found manganese reducers in manganese oxide-rich ocean sediments with different environmental conditions. These microorganisms were found to belong the Gamma- and Epsilonproteobacteria classes. However, manganese reduction is rarely tested in the description of new species, so that this capability might be more widespread in existing isolates than is currently known. As there is a large difference in redox potential between manganese and iron oxides, it seems likely that manganese reduction in the environment is carried out by specialized manganese reducers (Vandieken et al., 2012).

Mn(II)-oxidizing bacteria are widespread in environment and they can be isolated from almost all environmental samples. The physiological function of bacterial Mn(II) oxidation remains unknown. Because Mn(IV) formation is thermodynamically favorable, the bacteria could derive energy from the reaction; however, this has not been shown conclusively for any organism (Geszvain et al., 2012).

3. Radiotracers and their detection

3.1 Radioactive isotopes

The atomic number of an element identifies its chemical properties. The atomic number is the number of protons in the nucleus. Uncharged neutrons, within the nucleus along with protons, do not contribute to the atomic number, but will alter the atomic mass. The existence of the same element with different atomic weight makes them isotopes, variants of a particular element with different number of neutrons. Most isotopes are stable, and do not undergo any spontaneous nuclear changes. A subset of isotopes, possess too few or too many neutrons to be stable. These are radioactive isotopes or radioisotopes.

Radioisotope of a given element has the same number of electrons and shares a similar electronic structure as its stable element. Because the chemical behavior of an atom is mainly determined by its electronic structure, different isotopes exhibit nearly identical chemical behavior. Radioactive isotopes of common elements are useful in life science research, because radiolabelled compounds can substitute their nonradioactive compounds. The resulting radiolabelled compound is easily detectable but still chemically identical to the original material. Radioisotopes do not change during the chemical transformation of labeled molecules (Sorokin, 1999). The list of isotopes used in biological research is fairly small, for example, isotopes of carbon, hydrogen, oxygen, and phosphorus are widely used.

Intensity of isotopic radiation, radioactivity is measured as Bequerels (Bq). One Bequerel corresponds to a rate of one disintegration or decay per s or 60 per min. Radioactive decay is the process by which an unstable isotope emits energy to reach a more stable state. Radioactive material decays until only stable substance is left. The decay of a substance is fixed and measurable. Another commonly used unit of radioactivity is Curie and it is defined as 3.7 x 10^{10} disintegrations per second (dps). To convert from Bq to Ci and vice versa:

$$1\text{Bq} = 2.7 \times 10^{-11}\text{Ci} = 1\ \text{dps} = 60\ \text{dpm}$$

$$1\ \text{Ci} = 3.7 \times 10^{10}\ \text{Bq} = 3.7 \times 10^{10}\ \text{dps} = 2.22 \times 10^{12}\ \text{dpm}$$

Where

dps = disintegration per second

dpm = distintegration per minute

Disintegration per minute is directly related to the counts that one obtains in a radiometric detection instrument, e.g. liquid scintillation counter (cpm = counts per minute).

Radioactive decay occurs with the emission of particles or electromagnetic radiation from an atom due to a change within its nucleus. Radioisotopes release alfa or beta particles or gamma rays. Alfa particles consisting of two protons and two neutrons, are the least energetic, and most massive of these decay products. Alfa particles are able to travel only a few centimeters in air (Anonymous, 2004). Beta particles are high-energy electrons or positrons. There are two types of beta decay, known as beta minus and beta plus. An unstable atomic nucleus with an excess of neutrons may undergo beta minus decay, where a neutron is converted into a proton, an electron and an electron antineutrino. In beta plus decay a proton is lost and a neutron appears and the process produces a positron and electron neutrino. Depending on the beta particles energy (which depends on the radioactive atom), beta particles may travel up to several meters in air, and are stopped by thin layers of metal or plastic. Gamma -rays differ from alfa and beta emissions in that gamma -rays are electromagnetic radiation, not particles. Gamma -rays are quite penetrating, in many cases passing through up to 5 cm of lead (Anonymous, 2004).

The rate of radioactive disintegration does not depend on any physical or chemical condition, such as temperature, pressure, pH or redox potential, but is purely a statistical process. The half-life time is characteristic for each radioisotope and is a measure of the rate at which it decays to a nonradioactive state. Half-life is defined as the time it takes for a substance to decay sufficiently to convert half of its mass to a stable form. Half-lives may range from milliseconds to thousands of years but practical values are over ten days. The half-life of an isotope is fixed. The decay of a radioactive substance follows an exponential relationship:

$$N = N_0 e^{-ct}$$

Where

N = initial number of atoms of a given r**a**dioisotope at time t,

N_o = number of atoms at t = 0

c = decay constant

The decay constant is related to half-life of the given radioisotope (Sorokin, 1999).

3.2 ^{35}S radioisotope

Sulfur has four stable isotopes in nature ^{32}S, ^{33}S, ^{34}S, and ^{36}S their portions in nature being 95.02, 0.75, 4.21 and 0.02%, respectively. Sulfur has several radioactive isotopes of which others than ^{35}S (half-life 87.5 d) are relatively short-lived. ^{35}S is a weak beta emitter where an unstable atomic nucleus with an excess of neutrons undergoes decay. In ^{35}S decay a neutron is converted into a proton, an electron and an electron antineutrino. The beta particles are energetic electrons that move very quickly but can be stopped quite easily. E.g. ^{35}S is a low-energy beta emitter that has 26 cm range in air, 0.32 mm in water and tissue and 0.25 mm in plastic. Energy of ^{35}S is sufficient for nearly 100% detection efficiency by liquid scintillation counting, yet low enough to be unproblematic with the low activities used in tracer experiments generally. Its half-life is long enough to be insignificant during most incubations, yet short enough that the formed waste can be stored until the activity is below the limits for disposal as non-radioactive waste (Røy et al., 2014). In Figure 1 is shown a commercial ^{35}S labeled SO_4Na_2 package. Different amounts of labeled ^{35}S are available commercially.

Figure 1. On the left the outer cover packing and on the right the interior liquid container of the labeled $^{35}SO_4Na_2$.

3.3 Liquid scintillation counting

Liquid scintillation counting (LSC) is the main method by which long-lived low energy radioisotopes are quantitatively detected. LSC is used for measuring alfa

and beta particle activity in liquid radioisotope samples. The method is based on the detection of radioactive radiation by means of mixing sample material with liquid scintillation solution and counting the resultant photon emissions (Sorokin, 1999). Scintillation liquids contain an aromatic organic solvent, a surfactant and small amount of scintillators or fluors. In the sample the radioactivity e.g. beta particles emitted, cause solvent molecules to become excited. The energy of these solvent molecules is then transferred to the scintillators or fluors that in turn convert the energy emitted into light pulses. The light is detected and measured by the photomultiplier tube of the liquid scintillation counter. A phenomenon to be aware of in LSC measurement is quenching. Quenching is an energy loss occurring in the energy transfer processes. It causes a reduction in the photon yield and the detected (cpm) is reduced. Truly accurate measurement of disintegrations would require that every emission event would be detected and counted this is not possible in most situations. In addition, naturally occurring isotopes and cosmic radiation contribute significant background radiation that needs to be taken into account (Anonymous, 2004). However, scintillation counting is extremely sensitive relative to chemical measurements (Røy et al., 2014). Nevertheless, the produced LSC waste needs to be handled noticing its radioactive as well as toxic nature. In Figure 2 is presented a liquid scintillation counter.

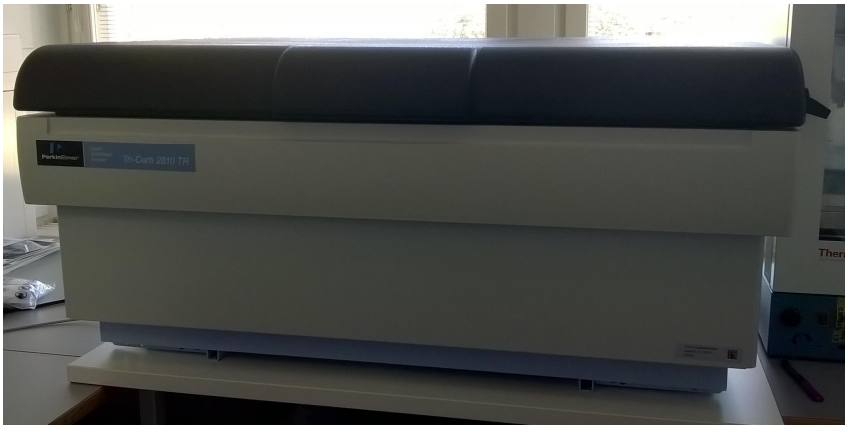

Figure 2. Liquid scintillation counter (Tri-Carb 2810TR Low Activity Liquid Scintillation Analyzer, PerkinElmer).

4. $^{35}SO_4^{2-}$-tracer analysis methods and applications in environmental microbiology

4.1 Development of $^{35}SO_4^{2-}$-tracer method for sulfate reduction rate measurements

Different $^{35}SO_4^{2-}$-tracer analysis methods have been developed with different drawbacks, advantages and specific applications. Anaerobic sulfate reduction produces sulfide, which may either remain in solution as hydrogen sulfide or precipitate as various forms of metals, mono- and disulfides, or elemental sulfur. All these inorganic end products are termed total reduced inorganic sulfur (TRIS) (Kallmeyer et al., 2004). The development of the $^{35}SO_4^{2-}$-tracer analysis methods started at 1957 by Ivanov who incubated the marine sediment with $^{35}SO_4^{2-}$-tracer anaerobically and separated the formed $H_2{}^{35}S$ with acid distillation (King, 2001). This basic method has been developed since then by others (e.g. Jørgenssen, 1978a & 1978b; Skyring, 1987). In acidic distillation the acid volatile sulfur (AVS) fraction including hydrogen sulfide and ferrous sulfide is released into N_2 gas phase where they are collected into zinc acetate trap as zinc sulfide. However, part of sulfide reactions such as formation of pyrite remains in the original sample and therefore the sulfate reduction may be underestimated (Howarth and Merkel, 1984).

Several options have been tested to assess the radioactivity in non-acid volatile reduced sulfur fractions. Elemental sulfur has been extracted with toxic solvents such as hexane, benzene or carbon disulfide (King, 2001). The use of aqua regia (a 1:1 mixture of concentrated hydrochloric and nitric acids) digestions have been tested to solubilize both elemental sulfur and pyrite in sediments but proved to be controversial. Boiling acidic chromous chloride distillation introduced by Zhabina and Volkov (1978) have proven more reliable and practical. Acidic chromous chloride reduces inorganic sulfur species, including pyrite but exclude sulfate, to hydrogen sulfide which is of the essence (King, 2001).

The hot acidic chromous chloride distillation step releases also pyrite and elemental sulfur. The AVS and chromium reducible sulfur (CRS) steps can be per-

formed in succession and different fractions can be analyzed separately or AVS and CRS steps can be combined into one step (Fossing and Jørgenssen, 1989). This method has yet a drawback as the recovery of the elemental sulfur depends on the crystallinity of the sulfur. In addition the method has inherent background problem as a minute amount of radioactivity distils over and creates a signal that cannot be attributed to sulfate reduction (Kallmeyer et al., 2004). However, this is not an issue as long as the sulfate reduction rate is high.

Kallmeyer et al. (2004) developed modified cold chromium distillation procedure for small amounts of radiolabeled reduced sulfur species for the determination of very low sulfate reduction rates. They reduced the amount of background radioactivity by lowering the distillation temperature and introduced the use of N,N-dimethylformamide (DMF) earlier used also by Hsieh and Yang (1989) to improve the recovery of elemental sulfur. The incubated and fixed sediment sample was centrifuged and two to three grams of sediment was transferred to a 3-neck round-bottom glass flask with 20 ml of DMF. The flask was flushed with N_2 for 10 min to avoid sulfide oxidation. Other chemicals were injected: 8 ml of 6N HCl and 16 ml of 1M $CrCl_2$. Sample mixture was bubbled with N_2 for 2 hours at room temperature in continuous stirring. The liberated sulfide was trapped as zinc sulfide in 7 ml of 5% ZnAc-solution with drop of antifoam. Between the zinc sulfide trap and the distillation flask was a citrate solution trap (7 ml 0.1 M citrate) to prevent aerosols from reaching the final ZnAc trap (Kallmeyer et al., 2004). The improved recovery of crystalline elemental sulfur was 74.8%. The addition of several blanks, time-zero blanks, sediment-free tracer blanks and counter blanks are important in successful application of this method in low sulfate reduction environments (Glombitza et al., 2013).

Røy et al. (2014) further modified this one step cold chromium distillation procedure. The main change was the replacement of toxic DMF with nontoxic dimethylsulfoxide (DMSO). However, DMF nor DMSO recover elemental sulfur totally, the recoveries vary between 50 to almost 100% (Kallmeyer et al., 2004; Røy et al., 2014). Other parameters that need to be considered for successful application of ^{35}S radioisotope methods especially in low sulfate reduction environments include several aspects. The incubation time should be kept relatively short as the depletion and recycling of tracer substrate should be minimized in particular if the original natural substrate concentration is low. However, if long incubation times are needed, for example due to low microbial activity, the incubations suffer from decay of radioactive TRIS and only little increase in TRIS is gained after more than 60 days. After 121 days, the decay of the TRIS pool will lead to decreased sensitivity (Røy et al., 2014). For the termination of ^{35}S incubation 5% or higher concentration of Zn-fixative is suitable and the best way to store the samples is to freeze them so that no oxidation of TRIS compounds occur (Røy et al., 2014).

To calculate the sulfate reduction rate (SRR in nmol /cm^3 d) in sediments the following equation is used (Kallmeyer et al., 2004).

$$SRR = [SO_4^{2-}] \times P_{SED} \times \left[\frac{A_{TRIS}}{A_{TOT}}\right] \times 1.06 \times \frac{1000}{t}$$

Where

$[SO_4^{2-}]$ = the sulfate concentration in the porewater (mmol/L)
P_{SED} = porosity of the sediment (mL porewater/cm^3 sediment)
A_{TRIS} = radioactivity of TRIS (cpm)
A_{TOT} = total radioactivity used (cpm or dpm)
t = the incubation time in days
1000 = factor for the change of units from mmol/L to $nmol/cm^3$

The factor 1.06 is an estimated isotope fractionation factor between $^{32}SO_4$ and $^{35}SO_4$ during bacterial sulfate reduction, which corrects for the slightly slower turnover of the heavy $^{35}SO_4$. The value is inferred from the ca. 40‰ mean fractionation in marine sediment between ^{32}S and ^{34}S (Canfield et al., 2010), under the assumption that the fractionation between ^{32}S and ^{35}S should be 50% larger (Røy et al., 2014). The equation is applicable to groundwater samples when the P_{SED} is set as one.

4.2 Sulfate reduction in marine and saline sediments

$^{35}SO_4^{2-}$-tracer methods have long been exploited in the measurements of dissimilatory sulfate reduction rates especially in marine sediments (Jørgensen, 1978a; Edenborn et al., 1987; Parkes et al., 1989; Kristensen et al., 2000; Glombitza et al., 2013). Marine environments with high sulfate concentration make sulfate a dominant electron acceptor in the anaerobic mineralization of organic matter in coastal marine sediments resulting in production of sulfide. The volume of $^{35}SO_4^{2-}$-tracer added into sample is small and contains only negligible amounts of sulfate not to change the sample's sulfate concentration and microbial steady state. The advantage of $^{35}SO_4^{2-}$-tracer method is, that only the added tracer is measured and the high background concentration of sulfate and sulfides in the sample is of little concern (Røy et al., 2014).

Sulfate reduction rates in different sediments have been measured using the whole core injection method of Jørgensen (1978a). Tracer amounts of the labeled substrate $^{35}SO_4^{2-}$ (aqueous solution) are injected into sediment core samples at multiple injection points through elastic seals in the wall of the coring tube. The whole core is incubated at *in situ* temperature for a few hours, the formed tracer sulfide is measured for each injected segment and the sulfate reduction rate is calculated. Zinc acetate solution is often used to stop the reaction after incubation. The reduced sulfur species are separated from the sediment by different hot or

cold acidic distillation methods and radioactivity is measured by liquid scintillation counting (Joye et al., 2004; Sawicka et al., 2012; Glombitza et al., 2013).

In marine estuaries the sediments with a high content of organic matter are generally highly reducing and sulfidic beneath an oxic and/or oxidized thin surface layer. The deposited organic matter is effectively buried in the zone dominated by bacterial sulfate reduction. Basically under the sulfate zone the buried organic matter remains to be degraded by methanogenesis that is the terminal pathway of organic carbon mineralization in anoxic environments (Jørgensen and Parkes, 2010). In anoxic marine sediments sulfate reduction accounts for up to 50% of the entire organic mineralization in coastal and shelf ecosystems where sulfate diffuses several meters deep into the sediment. Where sediments are high in organic matter, sulfate is depleted at shallow sediment depths, and biogenic methane production will occur (Plugge et al., 2011). In marine sediments, SRB may compete with methanogens or grow in syntrophy with methanogens depending on the prevailing environmental conditions (Muyzer and Stams, 2008). Also in freshwater ecosystems with sulfate concentrations of only 10–200 µM, sulfate is consumed efficiently within the top several centimeter of the sediments (Plugge et al., 2011). The sulfate reduction rates in marine anoxic sediments depend on e.g. salinity, sulfate concentration and organic load and its availability. The typical sulfate reduction rates in coastal surface-sediments are in the order of 20 nmol SO_4^{2-} cm^{-3} d^{-1} (Jørgensen, 1982).

4.3 Sulfate reduction in oligotrophic anoxic waters

Ekendahl and Pedersen (1994) studied nutritional responses of bacteria in deep subsurface groundwater from Stripa mine (Sweden). The bacteria grew into glass slides in laminar flow reactor connected to flowing anoxic groundwater with pH 9–10, from two levels (812–820 m and 970–1240 m below ground level) of a borehole. The numbers of attached bacteria reached 10^6–10^7 cm^{-2}. A glass slide was transferred under nitrogen atmosphere to a tube containing filtered groundwater. The radiolabelled substrate ($Na_2{}^{35}SO_4$) and in addition lactate was added to the tube. After incubation (1–4 h), the reaction was stopped, by precipitating H_2S with zinc acetate. Sulfur compounds were distilled with 1.5 ml of 5 M HCl which based on current knowledge distills only acid volatile fraction of sulfur compounds. No detectable production of sulfide was found in the samples with this method. Whether this is due to ineffective detection method or lack of microbial sulfate reduction in these conditions is impossible to assess.

The formation waters of deep repository of liquid radioactive waste site in Sevenyui, Estern Siberia, Russia were studied by Nazina et al. (2004). The region can be regarded as a small artesian basin that is open to another artesian basin. The weakly salty groundwater moves at velocities of 5–6 m y^{-1}. The repository has received waste since 1967, with approximately 2.8 x10^6 m^3 of waste with a total

activity of about 100 millions Ci disposed by 1998. The reservoir horizons are composed of weakly cemented sand–clay rocks and are separated by relatively impermeable clay strata. The radioactive waste has been injected into horizons 370 to 465 and 180 to 280 m below sea level. The wastes contain sodium salts (nitrates, acetates, carbonates, and sulfates), silica gel, and ions of several metals. The total salt content is up to 240 g L^{-1}. The relief wells have been drilled at the site to relieve the reservoir pressure and they are used to pump out subsurface waters simultaneously with waste injection. Samples of groundwater were collected directly from wellhead of relief wells and observation wells. Sulfate concentrations in formation waters varied from less than 4 to over 130 mg L^{-1} and the number of cultivable sulfate reducers from different water samples ranged from none to 100 cell per ml. To determine the bacterial sulfate reduction rate 50 ml bottles were completely filled with water and closed with butyl rubber stoppers and aluminium caps. Radioisotope solution was added to each bottle with a syringe. The incubation proceeded for 24 h at 24 °C. After incubation, the samples were inactivated with 1 ml of 40% NaOH and 0.5 mM of Na_2S was added to each bottle. The samples were acidified with 10% H_3PO_4 to pH 3, and hydrogen sulfide was distilled into a trap containing scintillation liquid. The measured sulfate reduction rates varied from 0.014 to 12.19 μg S^2 $L^{-1}d^{-1}$ (0.0004 to 0.4 nmol cm^{-3} d^{-1}). Some additional formation waters were analyzed later (Nazina et al., 2010) and in these samples the sulfate reduction rate varied from undetectable to 0.38 μg S^2 $L^{-1}d^{-1}$ (0.01 nmol cm^{-3} d^{-1}). Addition of waste components (acetate, sulfate, and nitrate) and molecular hydrogen to the samples of groundwater promoted sulfate reduction (Nazina et al., 2010).

Another deep sand horizon used as a repository for liquid radioactive wastes (LRW) at the Siberian Chemical Combine (Seversk, Tomsk oblast, Russia), was studied by Nazina et al. (2006). The reservoir horizons II and III are located at depths of 349–386 m and 270–320 m. Both reservoir horizons are composed for the most part of quartz (40–70%), clay minerals (10–40%), feldspar (5–20%); micas, chlorites, ferric oxides and hydroxides, carbonates, and sulfides were identified in small proportions. The groundwater flow rate is 3–5 m per year (Rybal'chenko et al., 1998). The system of injection wells is located in the central part of the repository; the observation wells are arranged concentrically around the injection contour. The radius of the repository is between 1.6 and 2.0 km. The low level wastes injected are slightly alkaline solutions (pH 6–10); salt concentrations vary between 0.2 and 40 g/l. The saline background consists of sodium salts of nitrates, hydrocarbonates, and sulfates. The total specific activity of solutions, ranges from 10^{-8} to 10^{-6} Ci L^{-1}. The number of cultivable sulfate reducers ranged from none to ten cell per mL in the studied samples. The rates of sulfate reduction in formation waters were determined by radioisotope method (Ivanov and Terebkova, 1959; Nazina et al., 2004) using labeled $Na_2{}^{35}SO_4$ and distillation with 10% H_3PO_4. The peak rate of sulfate reduction 0.1−0.55 μg S^{2-} $L^{-1}day^{-1}$ (i.e. 0.003 to 0.02 nmol cm^{-3} d^{-1}) was revealed in wells, located in the zone of waste dispersion.

In other water samples, the sulfate reduction rate did not exceed 0.05 µg S^{2-} $L^{-1}day^{-1}$ (Nazina et al., 2006).

In Perm, Russia, Pimenov et al. (2012) studied highly mineralized, sulfide- and sulfate-rich spring water for microbial diversity and sulfate reduction rate. After incubation with $Na_2{}^{35}SO_4$ solution for 1–3 days at 5–10°C, the samples were fixed with 1 mL of 2 N NaOH. The samples were then acidified with orthophosphoric acid and distilled under nitrogen flow in order to capture $H_2{}^{35}S$ by 2-phenylethylamine in the scintillation mixture. The number of microbial cells was only 5 x 10^4 cells per mL. However, the sulfate reduction rate of the unamended water was relatively high (0.575 mg S L^{-1} day^{-1} or 18 nmol cm^{-3} d^{-1}). From the enrichment cultures they detected sulfate-reducing microorganism (group *Desulfovibrio-Desulfomicrobium*) to dominate. No information on possible electron donors and organic carbon sources were given.

Sulfur compounds from the above three Russian study sites were acidified with phosphoric acid and distilled under nitrogen flow and captured straight into scintillation mixture. The results of this method are difficult to compare with the cold chromium distillation method (Kallmayer et al., 2004). With the phosphoric acid distillation method both relatively high and low sulfate reduction rates were measured.

Robador et al. (2015) studied the sulfate reduction with $^{35}SO_4{}^{2-}$-tracer method (Kallmeyer et al., 2004; Røy et al., 2014) in deep (2600 m bsl) basaltic ocean crust fluids from two drill holes. Incubation experiments were carried out with unamended fluids as well as with fluids amended with inorganic (H_2) and organic electron donors (a mixture of short-chain volatile fatty acids) at different temperature from 10 °C to 86 °C. They found that microbial sulfate reduction in organic acid-amended samples were consistently higher than in H_2-amended and unamended samples. They suppose that predominantly organotrophic sulfate-reducing communities in crustal fluids were inherently electron donor limited. They counted the cell-specific SRR range from 3.3 to 56 fmol $cell^{-1}$ day^{-1} in unamended experiments under *in situ* temperatures (39 and 63 °C).

5. Challenges and discoveries of environmental sulfur cycle research

In recent years so called cryptic sulfur cycle has been introduced in marine oxygen-minimum zones and sediment environments. It has been found out that after the sulfate reduction the formed sulfide may immediately oxidize back to sulfate and therefore the illustration of the sulfur reactions happening has been difficult to show (Canfield et al., 2015; Holmkvist et al., 2011; Reese et al., 2014). Active sulfate reduction is typically determined by the detection of sulfides. Microbiologically driven cryptic sulfur cycle where sulfide production is masked through sulfide re-oxidation back to sulfate, can be detected by identifying metabolically active sulfate reducing and sulfur oxidizing lineages co-locating within the studied environment. Geochemically driven cryptic sulfur cycle may exist when microbiologically produced sulfides react with iron and form rapidly iron sulfide minerals. The cryptic sulfur cycle has been proposed to be linked to anammox and other nitrogen cycling processes as well as to iron cycling in anaerobic environment (Canfield et al., 2015; Holmkvist et al., 2011; Reese et al., 2014).

Microbiological or geochemical methods interpreted independently may produce misleading conclusions from environmental samples due to the possible cryptic sulfur cycle existence (Reese et al., 2014). $^{35}SO_4^{2-}$-tracer analysis may help in the confirmation of cryptic sulfur cycle in case of formation of sulfide minerals. Separation of different formed sulfur compounds is not complete but acid volatile sulfur compounds (FeS and HS^-) and chromium reducible sulfur compounds (FeS_2 and S^0) is possible to distinguish from each other. If the microbial sulfide oxidation is expected to occur, labeled ^{35}S-sulfide can be used in tracer studies, however the labeled ^{35}S-sulfide has to be produced self. In a mixed microbial population including sulfate reducers, sulfide oxidizers and strains forming also intermediate products like S^0 and sulfite, the over all end products and production rates can be determined. However, results are a total outcome of different microbial and chemical reaction pathways combined together. Microbiological, environmental, chemical and geochemical variables probably introduce significant changes into such experiments. Nevertheless effects of individual variables can be studied. Tracer studies are always limited into laboratory conditions.

Another issue that should be taken into account when studying microbiological reactions is the existence of catabolic back fluxes. Especially reactions in environments where biochemical processes are close to thermodynamic equilibrium back fluxes are probable. It is possible that the measured fluxes of labeled substrates to products are not equal to microbial net rates (Holler et al., 2012). This is especially potential situation in oligotrophic low activity deep groundwater. $^{35}SO_4^{2-}$-tracer method does not detect this but the case needs to be checked with ^{35}S-sulfide experiment.

References

Amend, J.P. & Shock, E.L. 2001. Energetics of overall metabolic reactions of thermophilic and hyperthermophilic Archaea and bacteria. FEMS Microbiol. Rev. 25: 175–243.

Anonymous. 2004. Principles and applications of liquid scintillation counting. legacy.ehs.psu.edu/radprot/LSC_Theory2.pdf

Bomberg, M. & Itävaara, M. 2012. Olkiluoto groundwater fracture zones characterized by DNA and RNA based 16S rRNA-targeted 454 pyro sequencing and qPCR. Posiva working report 2012-27.

Bomberg, M., Lamminmäki, T. & Itävaara, M. 2015a. Estimation of microbial metabolism and co-occurrence patterns in fracture groundwaters of deep crystalline bedrock at Olkiluoto, Finland. Biogeosciences Discuss. 12: 13819–13857. doi:10.5194/bgd-12-13819-2015

Bomberg, M., Nyyssönen, M., Pitkänen, P., Lehtinen, A. & Itävaara, M. 2015b. Active microbial communities inhabit sulphate-methane interphase in deep bedrock fracture fluids in Olkiluoto, Finland. BioMed Res. Int. Article ID 979530. doi:10.1155/2015/979530

Böttcher, M.E. 2011. Sulfur cycle. In Reitner, J. & Thiel, V. (Eds.) Encyclopedia of geobiology. Springer. Pp. 859–864.

Canfield, D.E., Farquhar, J. & Zerkle, A.L. 2010. High isotope fractionations during sulfate reduction in a low-sulfate euxinic ocean analog. Geology 38: 415–418. doi:10.1130/ G30723.1

Canfield, D.E., Stewart, F.J., Thamdrup, B., De Brabandere, L., Dalsgaard, T., Delong, E.F., Revsbech, N.P. & Ulloa, O. 2015. A cryptic sulfur cycle in oxygen-minimum-zone waters off the Chilean coast. Science 330: 1375–1378.

Cole, J.A. & Brown, C.M. 1980. Nitrite reduction to ammonia by fermentative bacteria – short circuit in the biological nitrogen cycle. FEMS Microbiol. Lett. 7: 65–72.

Cord-Ruwisch, R., Seitz, H.-J. & Conrad, R. 1988. The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. Arch. Microbiol. 149: 350–357.

Cornell, R.M. & Schwertmann, U. 2003. The iron oxides – structure, properties, reactions, occurrence and uses. Weinheim: Wiley-VCH.

Das, A.P., Sukla, L.B., Pradhan, N. & Nayak, S. 2011. Manganese biomining: a review. Biores. Technol. 102: 7381–1387.

Edenborn, H.M., Silverberg, N., Mucci, A. & Sundby, B. 1987. Sulfate reduction in deep coastal marine sediments. Marine Chem. 21: 329–345.

Ekendahl, S. & Pedersen, K. 1994. Carbon transformations by attached bacterial populations in granitic groundwater from deep crystalline bed-rock of the Stripa research mine. Microbiology 140: 1565–1573.

Erguder, T.H., Boon, N., Wittebolle, L., Marzorati, M. & Verstraete, W. 2009. Environmental factors shaping the ecological niches of ammonia-oxidizing archaea. FEMS Microbiol Rev. 33: 855–869.

Finster, K., Liesack, W. & Thamdrup, B. 1998. Elemental sulphur and thiosulfate disproportionation by *Desulfocapsa sulfoexigens* sp. nov., a new anaerobic bacterium isolated from marine surface sediment. Appl. Environ. Microbiol. 68: 119–125.

Flynn, T.M., O'Loughlin, E.J., Mishra, B., DiChristina, T.J. & Kemner, K.M. 2014. Sulfur-mediated electron shuttling during bacterial iron reduction. Science 344: 1039–1042. doi: 10.1126/science.1252066

Fossing, H. & Jørgensen, B.B. 1989. Measurement of bacterial sulphate reduction in sediments. Evaluation of a single-step chromium reduction method. Biogeochemistry 8: 205–222.

Geszvain, K., Butterfield, C., Davis, R.E., Madison, A.S., Lee, S.-W., Parker, D.L., Soldatova, A., Spiro, T.G., Luther, G.W. & Tebo, B.M. 2012. The molecular biogeochemistry of manganese(II) oxidation. Biochem. Soc. Trans. 40: 1244–1248.

Ghosh, W. & Dam, B. 2009. Biochemistry and molecular biology of lithotrophic sulfu-roxidation by taxonomically and ecologically diverse bacteria and archaea. FEMS Microbiol. Rev. 33: 999–1043. doi: 10.1111/j.1574-6976.2009.00187.x

Glombitza, C., Stockhecke, M., Schubert, C.J., Vetter, A. & Kallmeyer, J. 2013. Sulfate reduction controlled by organic matter availability in deep sediment cores from the saline, alkaline lake Van (Eastern Anatolia, Turkey). Front. Microbiol. 4: Article 209. doi:10.3389/fmicb.2013.00209

Griebler, C. & Lueders, T. 2009. Microbial biodiversity in groundwater ecosystems. Freshwater Biol. 54: 649–677. doi:10.1111/j.1365-2427.2008.02013.x

Hedrich, S., Schlömann, M. & Johnson, D.B. 2011. The iron-oxidizing proteobacteria. Microbiology 157: 1551–1564.

Heimann, A., Jakobsen, R. & Blodau, C. 2010. Energetic constraints on H2-dependent terminal electron accepting processes in anoxic environments: a review of observations and model approaches. Environ. Sci. Technol. 44: 24–33. doi: 10.1021/es9018207

Hoehler, T.M., Alperin, M.J., Albert, D.B. & Martens, C.S. 1998. Thermodynamic control on hydrogen concentrations in anoxic sediments. Geochim. Cosmochim. Acta 62: 1745–1756.

Hoehler, T.M. & Jørgensen, B.B. 2013. Microbial life under extreme energy limitation. Nature Rev. 11, 83–94. doi:10.1038/nrmicro2939

Holmkvist, L., Gerdelman, T.G. & Jørgensen, B.B. 2011. A cryptic sulfur cycle driven by iron in the methane zone of marine sediment (Aarhus Bay, Denmark). Geochim. Cosmochim. Acta 75: 3581–3599.

Holler, T., Wegener, G., Niemann, H., Deusner, C., Ferdelman, T.G., Boetius, A., Brunner, B. & Widdel, F. 2012. Carbon and sulfur back flux during anaerobic microbial oxidation of methane and coupled sulfate reduction. PNAS. 108: E1484–E1490. doi:10.1073/pnas.1106032108/

Howart, R.W. & Merkel, S. 1984. Pyrite formation and the measurement of sulfate reduction in salt marsh sediments. Limnol. Oceanog. 29: 598–608.

Hsieh, Y.P. & Yang, C.H. 1989. Diffusion methods for the determination of reduced inorganic sulfur species in sediments. Limnol. Oceanogr. 34: 1126–1129.

Itävaara, M., Nyyssönen, M., Kapanen, A., Nousiainen, A., Ahonen, L. & Kukkonen, I. 2011. Characterization of bacterial diversity to a depth of 1500 m in the Outokumpu deep borehole, Fennoscandian Shield. FEMS Microbiol. Ecol. 77: 295–309.

Ivanov, M.V. & Terebkova, L.S. 1959. Study of microbial processes of H_2S formation in Lake Solenoe. Mikrobiologiya. 28: 251–256.

Jørgensen, B.B. 1978a. A comparison of methods for the quantification of bacterial sulphate reduction rates in coastal marine sediments. I Measurement with radiotracer techniques. Geomicrobiol. J. 1: 11–17.

Jørgensen, B.B. 1978b. A comparison of methods for the quantification of bacterial sulphate reduction rates in coastal marine sediments. Calculation from mathematical models. Geomicrobiol. J. 1: 29–47.

Jørgensen, B.B. 1982. Mineralization of organic-matter in the sea bed – the role of sulfate reduction. Nature 296: 643–645. doi:10. 1038/ 296643a0

Jørgensen, B.B. & Parkes, R.J. 2010. Role of sulfate reduction and methane production by organic carbon degradation in eutrophic fjord sediments (Limfjorden, Denmark). Limnol. Oceanogr. 55: 1338–1352. doi:10.4319/lo.2010.55.3.1338

Joye, S.B., Boetius, A., Orcutt, B.N., Montoya, J.P., Schulz, H.N., Erickson, M.J. & Lugo, S.K. 2004. The anaerobic oxidation of methane and sulfate reduction in sediments from Gulf of Mexico cold seeps. Chem. Geol. 205: 219–238.

Kallmeyer, J., Ferdelman, T.G., Weber, A., Fossing, H. & Jörgensen, B.B. 2004. A cold chromium distillation procedure for radiolabelled sulphide applied to sulphate reduction measurements. Limnol. Oceanogr. Met. 2: 171–180.

King, G.M. 2001. Radiotracer assays (^{35}S) of sulfate reduction rates in marine and freshwater sediments. Methods Microbiol. 30: 489–500. doi:10.1016/S0580-9517(01)30059-4

Kraft, B., Strousa, M. & Tegetmeyer, H.E. 2011. Microbial nitrate respiration – Genes, enzymes and environmental distribution. J. Biotechnol. 155: 104–117.

Kristensen, E., Bodenbender, J., Jensen, M.H., Rennenberg, H. & Jensen, K.M. 2000. Sulfur cycling of intertidal Wadden Sea sediments (Konigshafen, Island of Sylt, Germany): sulfate reduction and sulfur gas emission. J. Sea Res. 43: 93–104.

Lens, P. 2009. Sulfur cycle. In Schaechter, M. (Ed. in chief) Encyclopedia of Microbiology. Oxford: Elsevier. Pp. 361–369.

Lovley, D. & Chapelle F. 1995. Deep subsurface microbial processes. Rew. Geophys. 33: 365–381.

Lovley, D.R. & Goodwin, S. 1988. Hydrogen concentrations as an indicator of the terminal electron-accepting reactions in aquatic sediments. Geochim. Cosmochim. Acta 52: 2993–3003.

Lovley, D.R., Holmes, D.E. & Nevin, K.P. 2004. Dissimilatory Fe(III) and Mn(IV) reduction. Adv. Microb. Physiol. 49: 219–286.

Marietou, A., Griffiths, L. & Cole, J. 2009. Preferential reduction of the thermodynamically less favorable electron acceptor, sulfate, by a nitrate-reducing strain of the sulfate-reducing bacterium *Desulfovibrio desulfuricans.* J. Bacteriol. 191: 882–889.

Miettinen, H., Bomberg, M., Nyyssönen, M., Salavirta, H., Sohlberg, E., Vikman, M. & Itävaara, M. 2015. The diversity of microbial communities in Olkiluoto bedrock groundwaters 2009–2013. Posiva Working Report, 2015-12.

Mohan, S.B., Schmid, M., Jetten, M. & Cole, J. 2004. Detection and widespread distribution of the *nrf*A gene encoding nitrite reduction to ammonia, a short circuit in the biological nitrogen cycle that competes with denitrification. FEMS Microbiol. Ecol. 49: 433–443.

Muyzer, G. & Stams, A.J.M. 2008. The ecology and biotechnology of sulphate-reducing bacteria. Nature Rev. Microbiol. 6: 441–454.

Nazina, T.N., Kosareva, I.M., Petrunyaka, V.V., Savushikina, M.K., Kudriavtsev, E.G., Lebedev, V.A., Ahunov, V.D., Revenko, Y.A., Khafizov, R.R., Osipov, G.A., Belyaev, S.S. & Ivanov, M.V. 2004. Microbiology of formation waters from the deep repository of liquid radioactive wastes Severnyi. FEMS Microbiol. Ecol. 49: 97–107.

Nazina, T.N., Luk'yanova, E.A., Zakharova, E.V., Ivoilov, V.S., Poltaraus, A.B., Kalmykov, S.N., Belyaev, S.S. & Zubkov, A.A. 2006. Distribution and activity of microorganisms in the deep repository for liquid radioactive waste at the Siberian Chemical Combine. Microbiol. 75: 727–738.

Nazina, T.N., Safonov, A.V., Kodareva, I.M., Ivoilov, V.S., Poltaraus, A.B. & Ershov, B.G. 2010. Microbiological processes in the Severnyi deep disposal site for liquid radioactive wastes. Microbiol. 79: 528–537.

Nyyssönen, M., Hultman, J., Ahonen, L., Kukkonen, I., Paulin, L., Laine, P., Itävaara, M. & Auvinen, P. 2014. Taxonomically and functionally diverse microbial communities in deep crystalline rocks of the Fennoscandian shield. ISME J. 8: 126–138. doi:10.1038/ismej.2013.125

Parkes, R.J., Gibson, G.R., Mueller-Harvey, I., Buckingham, W.J. & Herbert, R.A. 1989. Determination of the substrates for sulphate-reducing bacteria within marine and estuarine sediments with different rates of sulphate reduction. J. General Microbiol. 135: 175–187.

Pereira, I.C., Abreu, I.A., Xavier, A.V., LeGall, J. & Teixeira, M. 1996. Nitrite reductase from *Desulfovibrio desulfuricans* (ATCC 27774) – a heterooli-gomer heme protein with sulfite reductase activity. Biochem. Biophys. Res. Comm. 224: 611–618.

Philippot, L. 2002. Denitrifying genes in bacterial and archaeal genomes. Biochim. Biophys. Acta 1577: 355–376.

Pimenov, N.V., Kuranov, G.V., Bryukhanov, A.L., Veslopolova, E.F., Koryukina, I.P. & Maslow Y.N. 2012. The sulfate-reducing bacterial community of sulfide-rich water of the Ust´-Kachka resort spring, Perm Krai, Russia. Microbiol. 81: 721–726.

Plugge, C.M., Zhang, W., Scholten, J.C.M. & Stams, A.J.M. 2011. Metabolic flexibility of sulfate-reducing bacteria. Front. Microbiol. doi:10.3389/fmicb.2011.00081

Reese, B.K., Witmer, A.D., Moller, S., Morse, J.W. & Mills, H.J. 2014. Molecular assays advance understanding of sulfate reduction despite cryptic cycles. Biogeochem. 118: 307–319.

Robador, A., Jungnluth, S.P., LaRowe, D.E., Bowers, R.M., Rappé, M.S., Amend, J.P. & Cowen, J.P. 2015. Activity and phylogenetic diversity of sulfate-reducing microorganisms in low-temperature subsurface fluids within the upper oceanic crust. Front. Microbiol. 5: 1–13. Doi: 10.3389/fmicb.2014.00748

Røy, H., Weber, H.S., Tarpgaard, I.H., Ferdelman, T.G. & Jørgensen, B.B. 2014. Determination of dissimilatory sulfate reduction rates in marine sediment via radioactive ^{35}S tracer. Limnol. Oceanogr. Methods 12: 196–211. Doi: 10.4319/lom.2014.12.196

Rybal'chenko, A.I., Pimenov, M.K., Kostin, P.P., Balukova, V.D., Nosukhin, A.V., Mikerin, E.I., Egorov, N.N., Kaimin, E.P., Kosareva, I.M. & Kurochkin, V.M. 1998. Deep Injection disposal of liquid radioactive waste in Russia. Columbus, Richland: Battelle Press.

Sawicka, J.E., Jørgenssen, B.B. & Brüchert, V. 2012. Temperature characteristics of bacterial sulfate reduction in continental shelf and slope sediments. Biogeosci. 9: 3425–3435. doi:10.5194/bg-9-3425-2012

Schleper, C. & Nicol, G.W. 2010. Ammonia-oxidising archaea – physiology, ecology and evolution. Adv Microb Physiol. 57: 1–41.

Schmidt, I. & Bock, E. 1997. Anaerobic ammonia oxidation with nitrogen dioxide by *Nitrosomonas eutropha*. Arch. Microbiol. 167: 106–111.

Schwertmann, U. & Fitzpatrick, R.W. 1992. Iron minerals in surface environments. In Skinner, H.C.W. & Fitzpatrick, R.W. (Eds.) Biomineralization processes of iron and manganese. Cremlingen: Catena. Pp. 7–31.

Skyring, G.W. 1987. Sulfate reduction in coastal ecosystems. Geomicrobiol. J. 5: 374.

Smith, C.J., Nedwell, D.B., Dong, L.F. & Osborn, A.M. 2007. Diversity and abundance of nitrate reductase genes (*nar*G and *nap*A), nitrite reductase genes (*nir*S and *nrf*A), and their transcripts in estuarine sediments. Appl. Environ. Microbiol. 73: 3612–3622.

Sohlberg, E., Bomberg, M., Miettinen, H., Nyyssönen, M., Salavirta, H., Vikman, M. & Itävaara, M. 2015. Revealing the unexplored fungal communities in deep groundwater of crystalline bedrock fracture zones in Olkiluoto, Finland. Front. Microbiol. 6: 573. doi: 10.3389/fmicb.2015.00573

Sorokin, Y.I. 1999. Radioisotopic methods in hydrobiology. Berlin Heidelberg: Springer-Verlag.

Straub, K.L. 2011a. Fe(II)-oxidizing prokaryotes. In Reitner, J. & Thiel, V. (Eds.) Encyclopedia of geobiology. Springer. Pp. 367–370.

Straub, K.L. 2011b. Fe(II)-oxidizing prokaryotes. In Reitner, J. & Thiel, V. (Eds.) Encyclopedia of geobiology. Springer. Pp. 370–373.

Straub, K.L., Benz, M. & Schink, B. 2001. Iron metabolism in anoxic environments at near neutral pH. FEMS Microbiol. Ecol. 34: 181–186.

Strous, M., Fuerst, J.A., Kramer, E.H.M., Logemann, S., Muyzer, G., van de Pas-Schoonen, K.T., Webb, R., Kuenen, J.G. & Jetten, M.S.M. 1999. Missing lithotroph identified as new planctomycete. Nature 400: 446–449.

Tebo, B.M., Johnson, H.A., McCarthy, J.K. & Templeton, A.S. 2005. Geomicrobiology of manganese(II) oxidation. Trends Microbiol. 13: 421–428.

Vandienken, V., Pester, M., Finke, N., Hyun, J.-H., Friedrich, M.W., Loy, A. & Thamdrup, B. 2012. Three manganese oxide-rich marine sediments harbour similar communities of acetate-oxidizing manganese-reducing bacteria. ISME J. 6: 2078–2090.

Wu, X., Holmfeldt, K., Hubalek, V., Lundin, D., Åström, M., Bertilsson, S. & Dopson, M. 2015. Microbial metagenomes from three aquifers in the Fennoscandian shield terrestrial deep biosphere reveal metabolic partitioning among populations. ISME J. doi:10.1038/ismej.2015.185

Yao, W. & Millero, F.J. 1996. Oxidation of hydrogen sulfide by hydrous Fe(III) oxides in seawater. Marine Chemistry 52: 1–16.

Zhabina, N.N. & Volkov, I.I. 1978. A method for determining various sulfur compounds in sea sediments and rocks. In Krumbein, W.E. (Ed.) Environmental Biogeochemistry and Geomicrobiology Vol. 3. Ann Arbor, Michigan: Ann Arbor Science. Pp. 735–745.

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Title	**^{35}S-tracer method for analyzing microbial sulfur compound cycling in oligotrophic anoxic groundwater habitat**
Author(s)	Hanna Miettinen
Abstract	Anaerobic, oligotrophic deep subsurface groundwater is an extremely demanding environment for microorganisms. This literature review presents an overview of microbial metabolism connected with dominant electron acceptors in such environments, including geological nuclear waste disposal repositories. The microbial activity is considerably lower in deep subsurface groundwater than in nutrient rich environments. The most common applicable terminal electron acceptors in this environment are nitrate, Mn(IV), Fe(III), sulfate, and CO_2 in the order of the decreasing energy yields. In deep geological repositories sulfate reduction to sulfide is considered as a major risk for safe disposal of high radioactive waste copper and steel capsules due to capsule biocorrosion. The literature review also outlines ^{35}S-label technique applicable to measure the rate of microbiologically produced sulfide. The general features of radioactive isotopes, their decay and detection are reviewed. Different kind of labeled sulfur distillation methods have been developed since 1950's. The applications of ^{35}SO4-tracer methods in environmental microbiology have started from ocean sediment sulfate reduction rate studies. At present distillation methods used are able to fractionate acid volatile sulfur compounds (FeS, HS^-) and chromium reducible sulfur compounds (FeS_2, S^0). ^{35}S-tracer methods are applicable also to study environments with low sulfate reduction rates, these studies are reviewed. Finally, problems associated with so called cryptic sulfur cycle in environment are summarized.
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Nimeke	**^{35}S-leiman käyttö mikrobiperäisten rikkiyhdisteiden analysoimiseksi vähäravinteisissa hapettomissa pohjavesissä**
Tekijä(t)	Hanna Miettinen
Tiivistelmä	Kallioperän hapeton, vähäravinteinen pohjavesi on vaativa elinympäristö mikrobeille. Kirjallisuuskatsauksessa esitetään yleiskatsaus mikrobien energia-aineenvaihdunnan kannalta tärkeimpiin elektronin vastaanottajiin tällaisissa ympäristössä, joita ovat mm. ydinjätteen loppusijoitustilat. Mikrobien aktiivisuus on huomattavasti matalampaa ja toiminta hitaampaa syvällä kallioperässä kuin ravinteikkaissa ympäristöissä. Yleisimmät hyödynnettävät elektronin vastaanottajat ovat nitraatti, Mn(IV), Fe(III), sulfaatti ja CO_2 laskevan energiasaannon mukaisessa järjestyksessä. Ydinjätteen loppusijoitustiloissa syvällä kallioperässä sulfaatin pelkistyminen sulfidiksi ja sen aiheuttama biokorroosio muodostavat huomattavan riskin radioaktiivisen jätteen kupari- ja teräskapselien kestävyydelle. Katsauksessa selvitetään radioaktiivisten isotooppien yleisiä ominaisuuksia, hajoamista ja hajoamisen määrittämistä sekä erityisesti leimatun rikin (^{35}S) ominaisuuksia ja käyttöä sulfidin muodostumisen mittauksessa. Erilaisia leimatun rikin tislaukseen perustuvia menetelmiä on kehitetty aina 1950-luvulta alkaen. Leimatun sulfaatin käyttö ympäristömikrobiologiassa on lähtenyt liikkeelle merisedimenttien sulfaatinpelkistysnopeuden määrityksistä. Nykyään käytössä olevissa sovelluksissa liuosnäytteistä voidaan erotella hapettuneen rikin eri yhdisteitä, kuten hapotuksessa haihtuvat yhdisteet (FeS, HS^-) sekä kromilla pelkistyvät rikkiyhdisteet (FeS^2, S^0). Tämän lisäksi uusimmat menetelmät ovat sovellettavissa aiempaa pienempien sulfaatinpelkistysnopeuksien määrittämiseen. Katsauksen lopussa käydään läpi kirjallisuudesta löytyviä tuloksia vähäravinteisten vesiympäristöjen sulfaatinpelkistysnopeuksista sekä ongelmista, jotka liittyvät niin kutsuttuun kryptiseen sulfaattikiertoon eri ympäristöissä
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^{35}S-tracer method for analyzing microbial sulfur compound cycling in oligotrophic anoxic groundwater habitat

This literature review presents an overview of microbial energy metabolism connected with geomicrobiological processes in deep subsurface groundwater and geological repositories. The most common applicable terminal electron acceptors for microorganisms in these environments are nitrate, Mn(IV), Fe(III), sulfate, and CO_2 in the order of the decreasing energy yields.

General features of radioactive isotopes, their decay and detection are reviewed. A ^{35}S-label technique, possible applicable for measurement of low amount of microbiologically produced sulfide that is corrosive for nuclear waste steel and copper capsules is more closely studied. At present ^{35}S distillation methods used are able to fractionate acid volatile sulfur compounds (FeS, HS^-) and chromium reducible sulfur compounds (FeS_2, S^0). Finally, problems associated with so called cryptic sulfur cycle in environment are summarized.

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