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Control of Microbial Activity by Engineered Barriers in Subterranean Waste Disposal

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ACADEMIC DISSERTATION
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PREFACE

ABSTRACT

The activity of microorganisms in subterranean waste disposal systems can be for good and bad in terms of environmental impacts of the waste disposal. The main objective of this thesis was to assess how the activities of methanotrophs (MOB) and sulfate reducers (SRM) can be affected by the characteristics of different soil and bentonite materials used in the engineered release barriers of municipal solid waste landfills and spent nuclear fuel (SNF) repositories. The other objective was to identify factors by which these microbial processes could be managed.

The MOB activity in landfill cover soils contribute to mitigation of CH_4 emissions. In the screened soils, the MOB activities were 10x higher in compost-based intermediate biocover soils than in mineral final cover soils. The activities correlated with the nitrate content and activity of heterotrophs in the soils, the latter of which was connected to soil organic matter (OM) content. The effect of different methods on increasing MOB activity in selected cover soils was assessed. An addition of compost to the soil (22 w-%) resulted in the greatest MOB activity increase due to facilitated diffusion of gases and increased nutrient content.

In SNF repositories, SRM produce sulfide that can corrode copper canisters sealing the SNF. The water-soluble OM released by the studied bentonites sustained the growth of SRM and other microorganisms at the simulated interface of compacted bentonite and bedrock, and the highest activities were associated with the use of Bulgarian bentonite in the experimental setup. The water-soluble OM quantity of all the bentonites was shown to be low relative to the total organic carbon content, even after being slightly increased by the simulated repository conditions (e.g., <20 w-%). The mineralogy of the bentonites (gypsum content and iron mineral composition) were also found to control the SRM activity.

To conclude, both MOB and SRM activities were shown to be dependent on the different physicochemical properties of the soils and bentonites, most of which could be managed by material selection. The findings of this work increased the understanding of controls of the specific microbial activities in the studied waste disposal barriers and, thus, can provide valuable information for planning and maintaining the barriers in practice.

TIIVISTELMÄ

Mikrobien aktiivisuus voi vaikuttaa sekä positiivisesti että negatiivisesti siihen, millainen ympäristövaikutus jätteiden maanalaisella loppusijoittamisella on. Tämän työn tavoitteena oli arvioida kuinka kaatopaikoilla ja käytetyn ydinpolttoaineen loppusijoituskohteessa käytettävien/käytettäväksi suunniteltujen materiaalien (maa, bentoniitti) ominaisuudet vaikuttavat metanotrofien (MOB) ja sulfaatinpelkistäjien (SRM) aktiivisuuksiin. Lisäksi tavoitteena oli tunnistaa tekijöitä, joiden avulla kyseisiä mikrobiprosesseja voitaisiin ohjata haluttuun suuntaan.

MOB:n aktiivisuus kaatopaikkojen pintamaassa pienentää niiden metaanipäästöjä. Tutkittujen pintamaa-ainesten osalta MOB:n aktiivisuus oli 10x korkeampi kompostipohjaisissa väliaikaisissa peitteissä kuin lopullisten pintarakenteiden epäorgaanisissa maa-aineksissa. Aktiivisuudet korreloivat maa-ainesten nitraattipitoisuuksien ja heterotrofien aktiivisuuksien kanssa, joista jälkimmäisellä oli yhteys maan orgaanisen aineksen (OM) pitoisuuteen. Työssä arvioitiin erilaisten menetelmien tehokkuutta MOB:n aktiivisuuden nostamiseksi valikoiduissa maanäytteissä. Kompostin lisääminen (22 m-%) osoittautui tehokkaimmaksi menetelmäksi, koska se paransi kaasujen kulkeutumista ja lisäsi ravinnepitoisuutta maa-aineksessa.

SRM:t tuottavat sulfidia, joka voi syövyttää käytettyä ydinpolttoainetta sisältäviä kuparikapseleita. Tutkituista bentoniiteista vapautuvan vesiliukoisen OM:n osoitettiin ylläpitävän SRM:n ja muiden mikrobien aktiivisuutta simuloidussa tiiviin bentoniitin ja peruskallion rajapinnassa ja korkeimmat aktiivisuudet havaittiin koelaitteistoissa, joissa käytettiin bulgarialaista bentoniittia. Vesiliukoisen OM:n pitoisuus bentoniiteissa oli matala suhteessa orgaanisen hiilen kokonaispitoisuuteen, myös siitä huolimatta, että simuloidut loppusijoituskohteen olosuhteet hieman nostivat sitä (<20 m-%). Bentoniittien mineralogian (kipsipitoisuus, rautamineraalien koostumus) havaittiin myös vaikuttavan SRM:n aktiivisuuteen.

Työn tulokset osoittavat, että sekä MOB:n että SRM:n aktiivisuudet ovat riippuvaisia maa-ainesten ja bentoniittien erilaisista fysikaaliskemiallisista ominaisuuksista, joista suurinta osaa voidaan ohjata materiaalin valinnan avulla. Työn tulokset ovat lisänneet tietämystä tekijöistä, joiden avulla voidaan vaikuttaa jätteiden maanalaisessa loppusijoituksessa käytettävien vapautumisesteiden mikrobiaktiivisuuksiin. Siten

tuloksia voidaan hyödyntää käytännössä vapautumisesteiden suunnittelussa ja ylläpidossa.

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ABBREVIATIONS

AGW	Artificial groundwater
Aq	Aqueous
As	Ämmässuo landfill
ATP	Adenosine triphosphate
BD	Below limit of detection
BR	Basal respiration
CBB	Calvin-Benson-Cycle
CEC	Cation exchange capacity
DIC	Dissolved inorganic carbon
DOC	Dissolved organic carbon
dw	Dry weight
EBS	Engineered barrier system
EC	Elimination capacity
Eh	Redox potential versus standard hydrogen electrode
EU-27	27 member states of the European Union
g	Gaseous
HA	Heterotrophic activity
HLW	High-level waste
ILW	Intermediate-level waste
INOC	The cell inoculated with microorganisms
IRB	Iron-reducing bacteria
KBS-3	"Kärnbränslesäkerhet", a technology for disposal of HLW developed by Swedish Nuclear Fuel and Waste Management Co.
L/S	Liquid-to-solid ratio
LLW	Low-level waste
LOI	Loss on ignition
MC	Moisture content
MLR	Multiple linear regression
MMO	Methane monooxygenase (s, soluble; p, particulate)
MO	Methane oxidation
MOB	Methane-oxidizing bacteria

MOR	Methane oxidation rate
MP	Methane production
MPN	Most probable number
MSW	Municipal solid waste
n	Number of observations
n.a.	Not applicable
n.m.	Not measured
n.r.	Not reported
OM	Organic matter
P	Phosphorus
PEEK	Polyether ether ketone
RE	Removal efficiency
RH	Relative humidity
RuMP	Ribulose monophosphate
s	Solid
scs	Silty clay soil
SD	Standard deviation
SE	Standard error
SEM/EDS	Scanning electron microscope/energy dispersive spectrometry
SeSl	Sewage sludge
SNF	Spent nuclear fuel
sOM	(Water-)soluble organic matter
SP	Swelling pressure
SRM	Sulfate-reducing microorganisms
SRR	Sulfate reduction rate
SS	Stainless steel
TDS	Total dissolved salts
TE	Trace elements
Tj	Tarastenjärvi landfill
TOC	Total organic carbon
TS	Total solids
TVO	Teollisuuden Voima Oyj
UNIC	The cell uninoculated with microorganisms
VLLW	Very low-level waste
WHC	Water-holding capacity
wt	Weight
ww	Wet weight

ORIGINAL PUBLICATIONS

This thesis is based on the following original research papers, which are referred to in this thesis by Roman numerals I–IV. The papers are reproduced with kind permissions of the publishers.

I   Maanoja, S. & Rintala, J. 2015. Methane oxidation potential of boreal landfill cover materials: The governing factors and enhancement by nutrient manipulation. Waste Management, 46: 399–407.

II   Maanoja, S. & Rintala, J. 2018. Evaluation of methods for enhancing methane oxidation via increased soil air capacity and nutrient content in simulated landfill soil cover. Waste Management, 82: 82–92.

III   Maanoja, S., Lakaniemi, A., Lehtinen, L., Salminen, L., Auvinen, H., Kokko, M., Palmroth, M., Muuri, E. & Rintala, J. 2020. Compacted bentonite as a source of substrates for sulfate-reducing microorganisms in a simulated excavation-damaged zone of a spent nuclear fuel repository. Applied Clay Science, 196: 105746.

IV   Maanoja, S., Palmroth, M., Salminen, L., Lehtinen, L., Kokko, M., Lakaniemi, A., Auvinen, H., Kiczka, M., Muuri, E. & Rintala, J. 2021. The effect of compaction and microbial activity on the quantity and release rate of water-soluble organic matter from bentonites. Submitted for publication in Applied Clay Science.

AUTHOR'S CONTRIBUTION

I   Susanna Maanoja wrote the manuscript and is the corresponding author. She planned and conducted the laboratory experiments and interpreted the results. Jukka Rintala participated in planning the experiments, interpretation of the results and revision of the manuscript.

II   Susanna Maanoja wrote the manuscript and is the corresponding author. She planned and conducted the laboratory experiments and interpreted the results. Jukka Rintala participated in planning the experiments, interpretation of the results and revision of the manuscript.

III   Susanna Maanoja wrote the manuscript and is the corresponding author. She planned the laboratory experiments with the co-authors and conducted the laboratory work mostly with Leena Lehtinen and Linda Salminen. Susanna Maanoja interpreted the results and wrote the first version of the manuscript with the help of Jukka Rintala and Aino-Maija Lakaniemi. The other co-authors participated in revision and editing the manuscript.

IV   Susanna Maanoja wrote the manuscript and is the corresponding author. She planned the laboratory experiments with the co-authors and conducted the laboratory work mostly with Leena Lehtinen and Linda Salminen. Susanna Maanoja interpreted the results and wrote the first version of the manuscript with the help of Jukka Rintala and Marja Palmroth. The other co-authors participated in revision and editing the manuscript.

1 INTRODUCTION

World population has increased from 2.5 to 7.8 billion people during the past 70 years and the growth is expected to continue throughout the 21st century, but at a decreasing rate (United Nations, 2019a, b). Along with growing population, developing economies and increased living standards, the global material and energy consumption have increased for example from 13.1 to 13.8 tons of material per capita and from 1290 to 1630 million tons of oil equivalents between 1970 and 2015 in Europe (UNEP, Nd.; Sadorsky, 2014; Ahmad & Zhang, 2020; Statista, 2020b). The economic growth and its by-products have also brought along consequences that have increased the environmental burden, including greenhouse gas emissions driving global warming and climate change, decrease of the biodiversity, and depletion of raw materials, food and water, among other impacts (Bilgen, 2014; Schandl et al., 2018; Ahmad & Zhang, 2020).

The increased material consumption has resulted in increased production of municipal solid waste (MSW) over the years, for example from 505 kg capita^{-1} year^{-1} in 1990 to 525 kg capita^{-1} year^{-1} in 2018 in the countries of Organisation for Economic Co-operation and Development (OECD, 2020). Landfilling of unsorted wastes in subterranean sites has been a common solid waste management practice in many countries around the world and it is still today executed in some countries, even though the reuse and recycling of the waste materials is pursued over disposal to landfills to an increasing extent in several countries (Korhonen et al., 2018; Sharma & Jain, 2020). As a result of landfilling unsorted organic waste, the organic material becomes decomposed microbiologically to methane (and CO_2) inside the landfills (Meyer-Dombard et al., 2020). Some of the produced methane may escape from the landfills to the atmosphere and the waste treatment sector is the third largest anthropogenic source of methane globally (IPCC, 2013).

Production of nuclear power started in the 1950s as a response to growing electricity demand and high price of the existing energy forms (Davis, 2012; Kónya & Nagy, 2018). Today, there are in total 440 nuclear reactors operating world-wide, and the share of electricity produced by nuclear power is approximately 10% (2700 TWh) of the total electricity produced globally (in 2019; IAEA, 2020; Statista, 2020a).

Nuclear power produces radioactive waste, which must be disposed safely (Bruno & Ewing, 2006). The total amount of high-level radioactive waste, or spent nuclear fuel (SNF), waiting for disposal was 22 000 m³ in 2013 (IAEA, 2018) and the only satisfactory solution to manage the high-level waste is by disposal in deep geological repositories (Birkholzer et al., 2012; Ewing, 2015). By sealing the SNF in copper/cast iron canisters surrounded by dense buffer bentonite at the depth of 250–1000 m of a suitable host rock, the possible environmental and safety impacts of the waste can be minimized (Birkholzer et al., 2012).

Microorganisms are the foundation of planet's ecosystem as they are responsible of cycling of organic matter and different elements and nutrients (Burgin et al., 2011) and, thus, they are present everywhere on Earth – in air, soil, water bodies, extreme environments and also in human (Gupta et al., 2017). The environmental conditions direct the evolution of microbial diversity in different environments. For example, MSW landfills can enrich and contain an abundant community of microorganisms tolerating different chemicals and conditions evolving with waste degradation (Stamps et al., 2016; Meyer-Dombard et al., 2020). The SNF repositories instead contain a mixture of microorganisms capable of utilizing whatever substrates become available; scarce organic matter, gases (H_2, CH_4) and abundant inorganic compounds (e.g., SO_4^{2-}) (Wolfaardt & Korber, 2012).

In the subterranean disposal of MSW and SNF, both beneficial and detrimental natural microbially-intermediated processes can take place. In landfills, naturally occurring methane-oxidizing bacteria (MOB) oxidize methane into CO_2 and water (Khlemenina et al., 2018), while in SNF repositories, the sulfate-reducing microorganisms (SRM) produce sulfide, which can ultimately cause corrosion of the SNF containers and release of radionuclides to the environment (Hall et al., 2020). By using different methods, the favorable natural microbial processes can be harnessed for human's needs and the unwanted ones can be suppressed (Gupta et al., 2017). Consequently, the methane oxidation activity of the MOB can be utilized for mitigating methane emissions from the landfills by engineered cover structures, while the sulfide producing activity of the SRM can be minimized by engineering the characteristics of the bentonite barrier surrounding the SNF (Scheutz et al., 2009; Wolfaardt & Korber, 2012). Thus, it is crucial to understand the interaction of the microorganisms and abiotic characteristics of the waste disposal sites and engineered structures for maximizing the pursued outcome, activity of MOB or inactivity of SRM in the release barriers (landfill covers and bentonite), for managing some of the environmental impacts of subterranean disposal of MSW and SNF.

2   BACKGROUND

2.1   Microorganisms in environment

Microorganisms are prokaryotic organisms divided into domains of *Bacteria* and *Archaea*, both of which started developing from the same ancestors of cellular organisms with evolving Earth approximately $3.8 \cdot 10^9$ years ago, bacteria forming their own branch earlier than archaea (Wächtenhäuser, 2006; Cavicchioli et al., 2019). The archaea have several similarities with the bacteria (e.g., prokaryotic cell structure) but they differ from them at the molecular level for example by cell wall composition and ribosomal characteristics (Prescott et al., 2005). The third domain, *Eucarya*, developed from the pre-cells of archaea at a later stage ($1.5 \cdot 10^9$ years ago) (Campbell & Farrell, 2006). The greatest difference between bacteria and archaea and the eukaryotic cells is that the former do not have a membrane-bound nucleus and they are smaller in size and have a simpler cell structure than eukaryotic cells (Wächtenhäuser, 2006; Gupta et al., 2017). Despite of being smaller and simpler than eukaryotic cells, bacteria and archaea possess more diverse metabolic capabilities and, thus, they have a crucial role in cycling elements and as a base of the food webs on the Earth (Fig. 1; Falkowski et al., 2008 ; Cavicchioli et al., 2019).
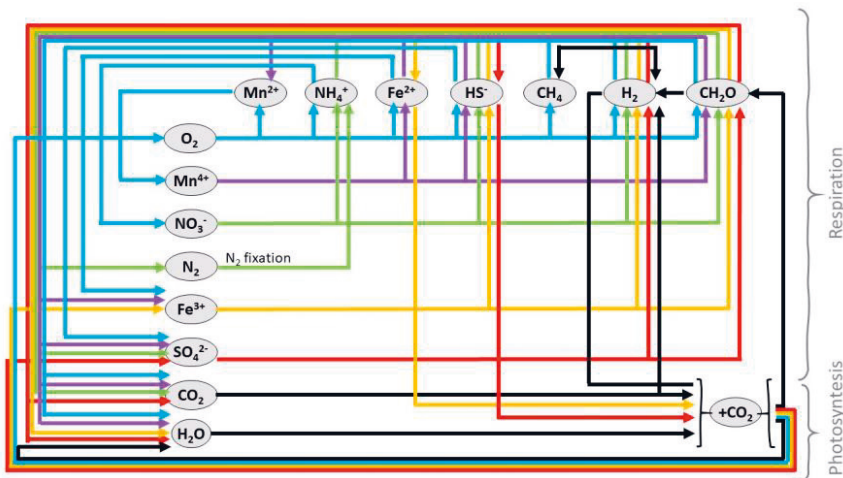
Figure 1.   Examples of microbially-mediated element cycles in the ecosystem (adapted from Falkowski et al., 2008)

The early ancestors of today's microorganisms were relying on chemoautotrophy to grow on the simple elements existing during that time (FeS, H_2S, CO, H_2), but since then the microorganisms have developed a range of metabolic reactions as a response to evolving conditions and substrates on the maturing Earth (Wächtenhäuser, 2006). The microbial energy producing metabolism is based on reactions, where electrons are transferred from electron donors to electron acceptors by oxidation, which results in reduction of the electron acceptor (Falkowski et al., 2008). The type of the compound the microorganism uses as an electron donor classifies the microorganisms as a chemotroph or phototroph (Burgin et al., 2011). The phototrophs use light as a source of energy, while the chemotrophs use either organic (organotrophs) or inorganic (lithotrophs) compounds as electron donors (Fig. 1; Burgin et al., 2011). The organotrophs can be further classified as autotrophs or heterotrophs depending whether the source of carbon is CO_2 or an organic compound, respectively (Prescott et al., 2005). In the presence of O_2, aerobic microorganisms dominate the microbial community, but in the absence of O_2, the group of organisms that possesses the highest energy yielding reaction in the prevailing conditions (e.g. pH) has an competitive advantage over the other microorganisms of the community (Fig. 2) (Falkowski et al., 2008; Helton et al., 2015). The energy yield in reduction of the terminal electron acceptors varies between different compounds; $O_2 > NO_3^- > Fe^{3+} > SO_4^{2-}$ (Burgin et al., 2011).
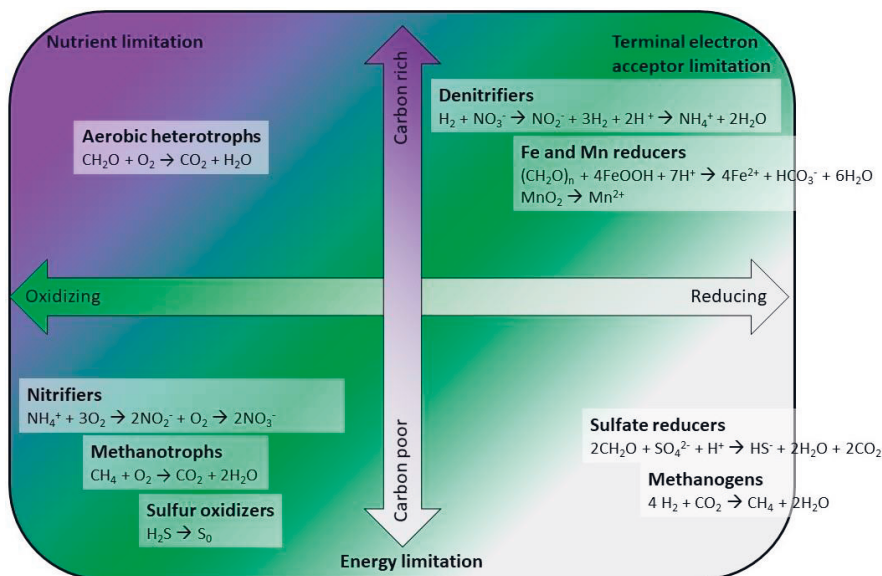
Figure 2. Examples of typical metabolic reactions of the microorganisms and the interaction of different factors controlling microbial activity (adapted from Gounot, 1994; Friedrich et al., 2001; Helton et al., 2015; Meyer-Dombard et al., 2020; Tuomi et al., 2020)

Microorganisms are ubiquitous in nature and they tend to spread everywhere (Falkowski et al., 2008) even including places intentionally made free of microorganisms by sterilization. Spreading of microorganisms is based on their vast distribution on the planet, and the transmittance of the microorganisms from surface or environment to another happens quickly by direct contact between contaminated and clean object or material or through air-transmission, as shown by several pathogens (Margesin & Miteva, 2011; Horve et al., 2020). As a response to spreading to environments with different and sometimes extreme conditions, the microorganisms have adapted their physiology at the molecular level to cope with the conditions (Blumer-Schuette et al., 2008). For example, thermophilic microorganisms can grow at 60–100 °C by synthetizing thermostable proteins and enzymes, and psychrophiles can grow at temperatures as low as –20 °C by synthetizing cold-active enzymes and antifreeze proteins among other physiological changes (Blumer-Schuette et al., 2008; Margesin & Miteva, 2011; Clarke et al., 2013; Gomes et al., 2016). Halophilic microorganisms instead can grow in environments with high salinity (up to ≥100 g total dissolved solids [TDS] L^{-1}) by increasing their cytoplasmic pressure to match with the extracellular osmotic pressure, while acidophiles cope with low pH (<3) by maintaining the intracellular pH around neutral with the help of specialized outer membrane functions (Oren, 2002; Sharma et al., 2016). The non-specialized microorganisms in less extreme environments have optimum growth conditions around 20–40 °C, pH 7±1, normal air pressure (1 atm) and adequate availability of water, nutrients, and salts (Satyanarayana et al., 2005).

The diversity of microorganisms is not only influenced by natural factors but also by human activities and the built environment (Jeffries et al., 2016; Horve et al., 2020). Examples of such human activities are contaminated environments (e.g. sediments; Burton & Johnston 2010) and for example different forms of waste disposal in subterranean repositories, as they modify the characteristics of the natural environment and introduce both harmful and utilizable substrates to the microorganisms (Stroes-Gascoyne et al., 1997; Jeffries et al., 2016; Stamps et al., 2016). In landfills, evolution of microorganisms is affected by the stage of the landfill (open, close), age and type of the waste, quantity and type of available substrates and nutrients, and climatic conditions (Meyer-Dombard et al., 2020). The intense decomposing activity of microorganisms quickly consumes O_2 inside the waste body, which leads to anaerobic conditions and gives rise to particularly methanogenesis, but also to many other anaerobic microbial reactions, depending on the substrates available (Fig. 2; Meyer-Dombard et al., 2020). In the SNF repositories, the natural anaerobic microbial community in the host rock is affected first by the construction

of the site, which leads to mixing of waters from different depths of the rock providing encounter of new microorganisms and substrates (Pedersen et al., 2014a), and then by the heat and radiation emitting from the emplaced SNF (initial temperature ≤100 °C, radiation 0.2–0.4 Gy h^{-1}) (Stroes-Gascoyne & West, 1997; Ranta-aho, 2008). Some of the microorganisms indigenous to bentonite might survive from the radiation emitting from the SNF thanks to high resistance towards radiation (total doses up to 30 kGy), while radiation is not assumed to shape the microbial community of the host rock as buffer bentonite prevents the radionuclides from escaping the canister (Posiva, 2012b; Wolfaardt & Korber, 2012). After closure of the repository and attenuation of the thermal and radioactive radiation of the SNF, the swelling pressure of the bentonite buffer, however, is expected to prevent the proliferation of microorganisms inside the bentonite (Stroes-Gascoyne et al., 2010; Posiva, 2012b). The evolution of microbial community at the interfaces of the bentonite and host rock instead is affected by the reducing conditions of the groundwater and different compounds dissolving from the bentonites (Wolfaardt & Korber, 2012).

The timescale considered in assessing site-specific evolution and environmental effects of waste disposal is significantly longer for the disposal of SNF (1 000 000 years) than for landfilling of MSW because of the slower decay of the radioactivity in SNF compared to gas and leachate production resulting from mineralization of MSW (up to 100 years post-closure) (Näslund et al., 2013; Bagchi & Bhattacharaya, 2015). When predicting the long-term evolution of the disposal sites, the climatic evolution must be considered. In the near-future, climate change is expected to induce increased atmospheric temperature and CO_2 concentration, eutrophication of water bodies and great variation in soil moisture conditions (Cavicchioli et al., 2019; Meyer-Dombard et al., 2020). The effect of increased atmospheric temperature on temperature-sensitive MOB is of a great concern for continuum of methane emission mitigation from the landfills (Meyer-Dombard et al., 2020). In the repository environment, the dilute meteoric and glacial melting waters infiltrating to the repository depth (starting from approximately 10 000 and 50 000 years after closure, respectively) can decrease the salinity, introduce new substrates for the microorganisms (e.g. carbonates) and decrease the bentonite density through chemical erosion (Posiva, 2012b; Hellä et al., 2014). All these consequences could affect the evolution of microbial community in the repository.

Understanding how both short- and long-term environmental conditions and factors affect the mobility, occurrence and activity of microorganisms is crucial for managing the microbially-mediated environmental effects of the different forms of

subterranean waste disposal. Thus, in the following, the typical structures in subterranean disposal of MSW and SNF are described (Section 2.2), and the current knowledge on the selected microbial processes (methane oxidation and sulfate reduction) and the factors affecting the activity of corresponding microorganisms in the subterranean waste disposal structures is reviewed (Section 2.3).

2.2   Structures in subterranean waste disposal

Subterranean disposal is the current method planned to be used for managing SNF and it also has been used for managing MSW in the past, but to a decreasing extent today, at least in the European countries (Alley & Alley, 2014; Chen et al., 2020). The type of the disposed waste governs the design of the waste disposal site excavated at varying depths below the ground (landfills up to 100 m, SNF repositories 200–1000 m; Birkholzer et al., 2012; Meyer-Dombard et al., 2020). Depending on the waste, the site-specific structures can be designed either to completely confine the waste to the repository without an interaction with the surrounding environment (SNF) or then to allow a flow of material (e.g. gas, leachate) out from the waste body through engineered structures for separate treatment (MSW landfills) (Posiva, 2012b; Cossu, 2018). The structures controlling the movement of different materials in landfills and deep geologic repositories are called release barriers and they have a crucial role in minimizing the environmental effects of the subterranean waste disposal as described in the following sections.

2.2.1   Municipal solid waste landfills

In European countries (EU-27), one person produces approximately 490 kg of MSW per year resulting in total 219 million tons of waste annually (inspection period 1995–2018; Eurostat, 2020). The MSW typically consists of source-separated or unseparated wastes collected from households, institutes and commercial premises and, thus, is composed of 36% organic wastes (food and garden residues), 19% paper and cardboard, 12% plastic waste, 8% glass, 3% metal and 23% rubber, leather, wood and other wastes (countries of Europe and Central Asia in 2016; Kaza et al., 2018). Even though an increasing proportion of the MSW produced is recycled in the European countries (EU-27), a small share is still being disposed to landfills (11% or 52 million tons in 2018; Eurostat, 2020). In Europe, the share of the landfilled waste

has decreased drastically during the past decades as for example in 1995, the share of landfilled MSW was 61% (121 million tons; Eurostat, 2020). The separation of biodegradable waste from the MSW was started in early 2000s after the Landfill Directive (1999/31/EC) came into force but before that it was generally landfilled with the other MSW fractions (EEA, 2013).

In landfills, decomposition of organic material starts with aerobic microbial processes and is followed by anaerobic processes after depletion of O_2 (Cossu et al., 2018). As a result of anaerobic microbial degradation, the landfilled organic waste is transformed mainly to CO_2 and methane, whose concentration in the landfill gas is 30–60% and 40–70% (v/v), respectively (Abbasi et al., 2012). The production of landfill gas is at its highest 5–7 years after closure and it lasts for few decades after the closure (ATSDR, 2001). For example, a MSW landfill established in 1989, which stopped receiving organic waste in 1997, still produced 26 kg CH_4 d^{-1} after approximately 25 years (Cassini et al., 2017). Both CO_2 and methane are greenhouse gases contributing to global warming, but methane's capability of absorbing and emitting thermal radiation is approximately 28x higher than that of CO_2 (in terms of a 100-year-projection) and, therefore, the emissions of methane from the landfills should be prevented (Höglund-Isaksson et al., 2012; IPCC, 2013). Contribution of landfilling and other waste treatment sectors (e.g. wastewater, manure treatment) to global methane emissions (approximately 556 Tg CH_4 $year^{-1}$ in 2011) was 12–16%, which corresponds to approximately one fifth of the emissions from anthropogenic sources (354 Tg CH_4 $year^{-1}$ in 2011; IPCC, 2013).

A modern MSW landfill that qualifies the requirements of the Landfill Directive (1999/31/EC) contains typically a basal impermeable mineral layer (e.g. composite, natural clay), leachate and other drainage systems (e.g. for groundwater), the waste body and a gas collection system among other smaller yet as important structures, and after the landfill has been closed, also artificial sealing and impermeable mineral layers, a surface water drainage layer and a top soil cover (Fig. 3a; EPA, 2000). In addition to the modern landfills described above, a great variety of other kinds of landfills exist globally ranging from illegal open dumps to legal landfills that can be either completely closed (dangerous waste), partially closed, or completely open (treatment of gaseous emissions and aqueous leachates required) (Damgaard et al., 2011). In engineered landfills, the generated methane along with landfill gas is eliminated by utilizing the chemical energy of methane by valorization or burning it without energy recovery after collection (Ménard et al., 2012). However, these strategies become ineffective and expensive to maintain when the concentration of methane in the landfill gas decreases below 20–30% and flow falls below 10–50 m^3

h[-1] (Huber-Humer et al., 2008; Ménard et al., 2012). In addition, the flaring and gas collection systems are not convenient to build or economical to maintain in small or remotely located landfills (Ménard et al., 2012) and the gas collection systems might also leak, so another application is needed for hindering the minor methane emissions (Humber-Humer et al., 2008; Yazdani et al., 2015).
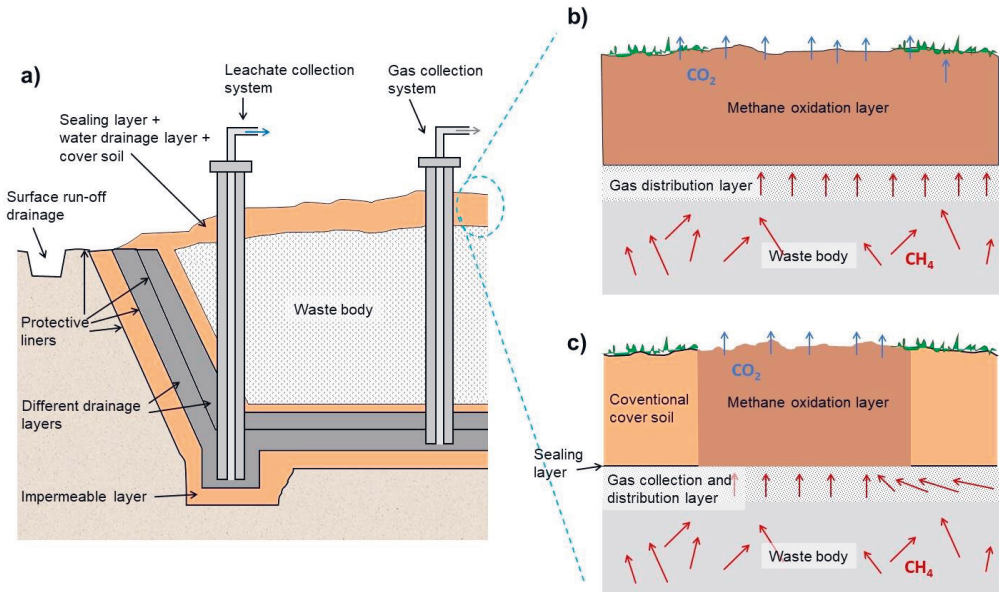
Figure 3. a) Schematic of the landfill structures, b) biocover and c) biowindow supporting activity of methane-oxidizing bacteria (not in scale; adapted from EPA, 2000; Kjeldsen & Scheutz, 2018; Artiola, 2019)

Methane emissions from landfills can be mitigated by utilizing biological methane oxidation taking place naturally in the soil covers of the landfills (Henneberger et al., 2015). Entire or partial soil covers especially designed to support methane oxidation, called biocovers and biowindows, can be installed at the surface of the landfills and they can be either for permanent or interim use (Fig. 3b, c; Kjeldsen & Scheutz, 2018). A permanent biocover consists of engineered structures containing gas distribution layers and layers of material supporting methane oxidation (e.g., compost) in the cover (Fig. 3b, c), while an intermediate biocover consists of about 30–50 cm deep layer of mixed soil materials (fine stone aggregates, compost) scattered directly on the waste and which can be left in place when a new layer of waste is applied (EPA, 2000). The operation period for an intermediate cover ranges from few days to several years (Huber-Humer et al. 2008). In addition to the structures supporting methane oxidation, the soil cover of a landfill can be

constructed solely for landscaping purposes after landfilling is terminated (Nochian et al., 2019). In that case, the accessible and available spoils and mineral soils are often used with minimum recommended height of 1 m (1999/31/EC; EPA, 2000; Sadasivam & Reddy, 2014). The bacteria responsible of methane oxidation, MOB, and the factors affecting their activity in different landfill cover materials are reviewed in Section 2.3.1.

2.2.2 Deep geological repository of spent nuclear fuel

The waste generated in production of nuclear electricity can be divided into very low- (VLLW), low- (LLW), intermediate- (ILW) and high-level radiation-emitting waste (HLW) based on the activity concentration of radionuclides (STUK, 2017). VLLW ($\leq$100 kBq kg^{-1}) and LLW ($\leq$1 MBq kg^{-1}) consist of slightly contaminated items, such as tools, clothing and laboratory gear, and they do not require special safety arrangements in terms of radioactivity for disposal (STUK, 2017; Kónya & Nagy, 2018). ILW ($1 < x \leq 10$ GBq kg^{-1}) consists of by-products from reactor operation, e.g. ion exchange resins and filters and products from reprocessing and decontamination of the HLW, while HLW comprises mostly of vitrified high-level waste and SNF (uranium pellets, UO_2; 1000 GBq kg^{-1}) (IAEA, 2007; Ewing, 2015; STUK, 2017). Globally, the amount of SNF to be disposed is 22 000 m^3 (in 2013; IAEA, 2018) while in Finland, the amount of radioactive waste is expected to be 8.3 · 10^3 tons of uranium and 3.1 · 10^3 m^3 of other waste covering the entire expected service time (60 years) of the existing and planned reactors (TEM, 2015). Radioactive wastes, in particular SNF, are dangerous due to the radioactivity and they must be processed and disposed in a way that they do not cause harm to humans or the environment (STUK, 2017).

The HLW is stored temporarily in facilities near the nuclear power plants, but due to an increasing amount of the waste produced, there is a need to find a solution for final disposal (Alley & Alley, 2014). The geologic disposal has been internationally identified as the preferred end-point method for managing the SNF because it meets the requirements of long-term passive containment of the radiation (Falck & Nilsson, 2009; Kónya & Nagy, 2018). Reprocessing of the SNF by dissolution and separation of the fission products has been suggested as an alternative waste treatment method, but it produces LLW and ILW among other side products and, thus, does not offer a permanent end-point solution leaving geologic disposal the only realistic method existing (Birkholzer et al., 2012; Kónya &

Nagy, 2018). However, finding a suitable location for the repository is challenging due to the strict requirements set for the features of the natural system (Falck & Nilsson, 2009; Alley & Alley, 2014). The soil formation hosting a SNF should be geologically stable and have a low water conductivity and stable geochemical and hydrochemical conditions to protect the repository from geological changes and to retard radionuclide transportation (Birkholzer et al., 2012). Therefore, crystalline, clay-based, and volcanic rocks and salt have been proposed as host rocks for the repositories in different countries of the world (Birkholzer et al., 2012). In Finland, several locations were screened for their geological and hydrogeochemical characteristics, and eventually the island of Olkiluoto in Eurajoki was selected because of suitable geology of the bedrock and the proximity of the source of the waste and the disposal site and the close co-operation of the Eurajoki municipality and TVO, the nuclear power company, among other reasons (Kojo, 2009; STUK, 2017).

The subterranean disposal of SNF has been planned in several countries around the world and the disposal concepts vary according to the local natural settings, but in all concepts the environmental impacts of disposing the SNF are attempted to be minimized by following a multi-barrier principle (Birkholzer et al., 2012; Ewing, 2015). In Finland, the KBS-3 concept developed by the Swedish Nuclear Fuel and Waste Management Co. is planned to use for disposal of SNF (Fig. 4; Posiva 2012a). The crystalline bedrock has a role as the outer-most natural barrier for the repository, which consists of several horizontal tunnels with vertical deposition holes mined at the depth of 400–450 m below the ground (Posiva, 2012a). The engineered barrier system (EBS) includes a copper/cast-iron canister and buffer and backfill bentonites (Posiva, 2012a). The SNF pellets are packed into rods, which are sealed inside the canisters (Posiva, 2012a). The canisters are placed in the deposition holes and embedded in buffer bentonite comprising of compacted blocks and pellets (Posiva, 2012a). Lastly, the tunnels leading to the deposition holes are backfilled with bentonite blocks and pellets to keep the contents of the deposition holes in place, and eventually each tunnel is sealed with a concrete-based plug (Posiva, 2012a). The repository is expected to fulfill its performance targets for up to 1 000 000 years (Posiva, 2012b). The performance cannot, thus, be predicted only with the help of empirical data often, which is obtained over limited (short) time periods, so modelling of the prevailing processes and data obtained from the natural analogs are jointly used for assessing the performance (Poinssot & Gin, 2012).
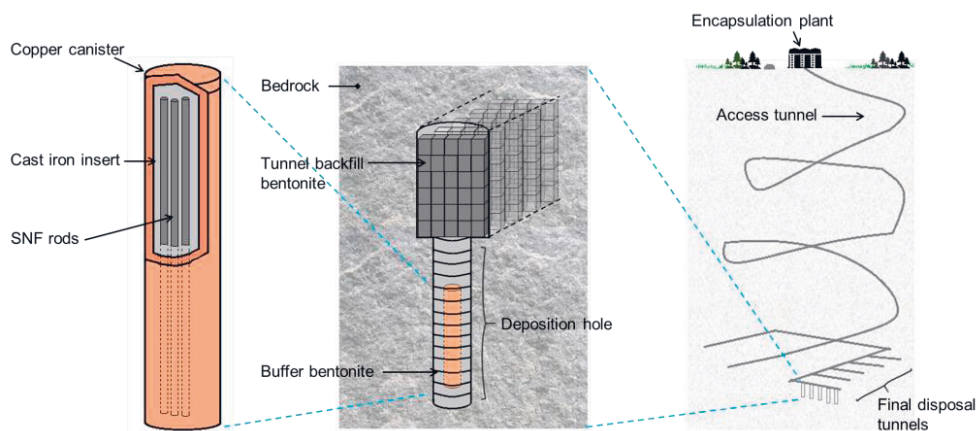
Figure 4. Schematic of a spent nuclear fuel (SNF) disposal system (not in scale; adapted from King et al., 2021)

The main function of the different EBS components is to protect the canister containing the SNF from geological and chemical changes in the prevailing conditions and to prevent the release of radionuclides to the environment (Posiva, 2012b; Ewing, 2015). Sulfide produced by the SRM inhabiting the groundwater systems has been recognized as one of the main agents causing corrosion of the copper canisters in the repository environment (Pedersen et al., 2008; King et al., 2012). Thus, one important safety function of the buffer bentonite is to prevent the diffusion of sulfide and other corroding agents to the canister surface and to inhibit the activity of SRM in the vicinity of the canister (Posiva, 2012b; Juvankoski, 2013). These targets are achieved by installing the bentonite buffer at an average dry density of 1595 kg m^{-3} (average saturated density of 2000 kg m^{-3}) (Juvankoski, 2013). When the bentonite becomes in contact with groundwater at the interfaces of the host rock, it swells and attains a swelling pressure of 7 MPa and hydraulic conductivity of <1 · 10^{-12} m s^{-1} limiting the microbial activity and transport of chemical agents (Posiva, 2012b; Juvankoski, 2013).

The swelling and self-healing characteristics of the bentonites can be attributed to the behavior of the main mineral of bentonite, montmorillonite (75–90% w-% requirement for buffer) belonging to a mineral group of smectites (Murray, 2006; Juvankoski, 2013). Montmorillonite consists of layers of negatively charged structural sheets consisting of two silica tetrahedral sheets encasing one alumina octahedral sheet (T-O-T; Fig. 5; Murray, 2006). The layers are held together by hydrated exchangeable cations (e.g. Na$^+$, Ca^{2+}) and different chemical and electrostatic forces and bonds in the interlayers (Fig. 5; Murray, 2006). The stacked layers form particles, which again form aggregates mixed with a small fraction of

different accessory minerals (e.g. gypsum, calcite, pyrite; Fig. 5; Bradbury & Baeyens, 2003; Perdrial & Warr, 2011; Juvankoski, 2013). Swelling of the montmorillonite is mainly governed by hydration of the interlayer cations causing expansion of the interlayers, and to a lower extent by electrical double-layer repulsion at the particle surfaces (Bradbury & Baeyens, 2003; Perdrial & Warr, 2011). In confined space, build-up of osmotic pressure between the ionic solution of fully swollen interlayers and the external water source creates the swelling pressure intended to inhibit microbial activity (Karnland et al., 2006; Posiva, 2012a). However, if the density (and swelling pressure) of the installed bentonite decreases for example locally because of mechanical erosion, the activity of SRM in the bentonite is possible (Stroes-Gascoyne et al., 2011). The effect of bentonite density and other factors affecting the biological sulfate reduction in the expected repository conditions are reviewed in Section 2.3.2.
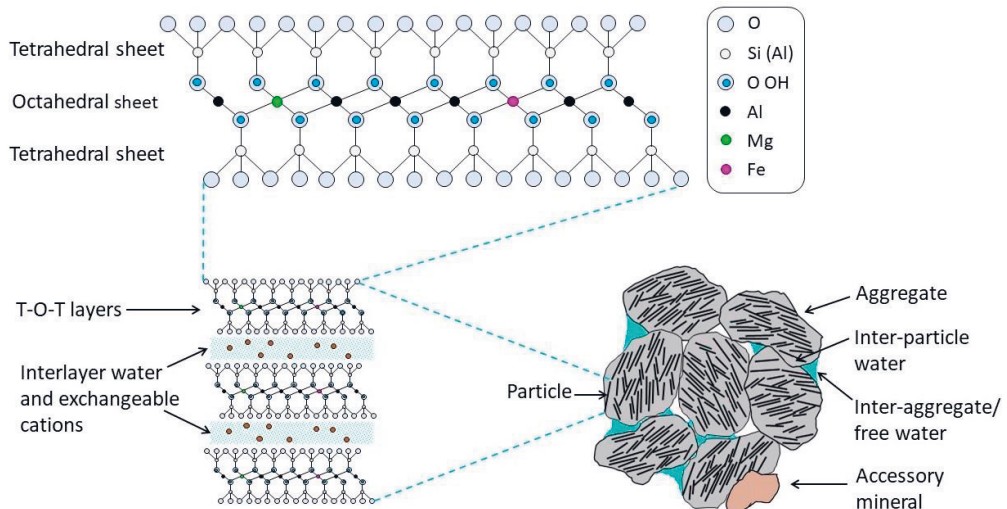
Figure 5. Schematic of montmorillonite layer, particle, and aggregate structures (adapted from Bradbury & Baeyens, 2003; Perdrial & Warr, 2011; Birgersson et al., 2017; Navarro et al., 2019)

2.3   Microbial activity in subterranean waste disposal structures

The environmental threats of subterranean disposal of MSW and SNF, controlling of which are in the focus of this thesis, include microbially contributed emission of methane and sulfide-induced corrosion of copper, respectively. The activity of MOB in the landfills and activity of SRM in the SNF repository have a crucial role in

mitigating the methane emissions and producing the copper-corroding sulfide, respectively (Scheutz et al., 2009; Stroes-Gascoyne et al., 2010). In the following (Sections 2.3.1 and 2.3.2), the current knowledge on these specific microbial processes and the factors affecting the activity of the microorganisms in the MSW and SNF disposal systems are reviewed.

Methane oxidation and sulfate reduction are not explicit to MSW landfills and SNF repositories, respectively, but they co-exist in both disposal systems. In landfills, sulfate reduction takes place in anaerobic parts of the waste body and it contributes to the malodorous emissions arising from landfills (Jin et al., 2020). The sulfuric emissions can be effectively mitigated by several methods, including active exhaustive gas management and biological sulfur oxidation, which can be maintained simultaneously with methane oxidation in landfill cover structures (McKendry et al., 2002; Lee et al., 2018; Rossi et al., 2020). In the SNF repositories, both aerobic and anaerobic methane oxidation may occur consuming the methane in the groundwater (Bomberg et al., 2015; Kietäväinen & Purkamo, 2015). Aerobic methane oxidation is considered as one of the beneficial oxygen-consuming biological processes in the SNF repository especially post-closure as O_2 has a potential to corrode copper (Chi Fru, 2008; King et al., 2017). The anaerobic methane oxidation can be carried out in syntrophic consortia of anaerobic methanotrophs and SRM, but no evidence has been shown that this process would significantly contribute to the sulfide inventory of the repository system (Rabus et al., 2015; Tuomi et al., 2020).

2.3.1   Methane oxidation in landfills

Aerobic MOB possess a unique ability to use methane as a sole source of carbon and energy and they naturally inhabit locations at the interface of aerobic and anaerobic aquatic and terrestrial environments near methane sources, such as in those existing at landfills (Hanson & Hanson, 1996; Su et al., 2014). MOB are a vast group of bacteria that belong mainly to the subdivisions α and γ of proteobacteria phylum and also to a non-proteobacterial phylum *Verrucomicrobia* (Khlemenina et al., 2018). While carrying out the oxidation reaction MOB transform methane into CO_2 and biomass (Fig. 6; Murrell, 2010). They can be divided into two main types, I and II (minor types X, III and IV), according to the metabolic pathway they utilize to assimilate the methane-derived carbon (Fig. 6; Kalyuzhnaya et al., 2015). The first by-product of methane oxidation reaction is methanol, which is further oxidized to formalde-hyde, then to formic acid, methylene and eventually to CO_2 (Fig. 6; Kalyuzhnaya et

al., 2015). The difference between the pathways is that type I bacteria utilizing ribulose monophosphate (RuMP) pathway assimilate almost all of their carbon from formaldehyde, whereas type II bacteria utilizing the serine pathway receive only part of their carbon from formaldehyde and the rest from CO_2 (Yang et al., 2013; Hakobyan & Liesack, 2020). In addition to the different metabolic pathways, different MOB species differ from each other in the expressed enzymes. Methane monooxygenase (MMO) is an enzyme catalyzing oxidation of methane to methanol (Fig. 6; Kalyuzhnaya et al., 2015). Some type II MOB and a few type I MOB have reported to express only the soluble form of MMO (sMMO), while almost all the MOB express the particulate, membrane-bound form of the enzyme (pMMO) (Murrell, 2010; Hakobyan & Liesack, 2020).
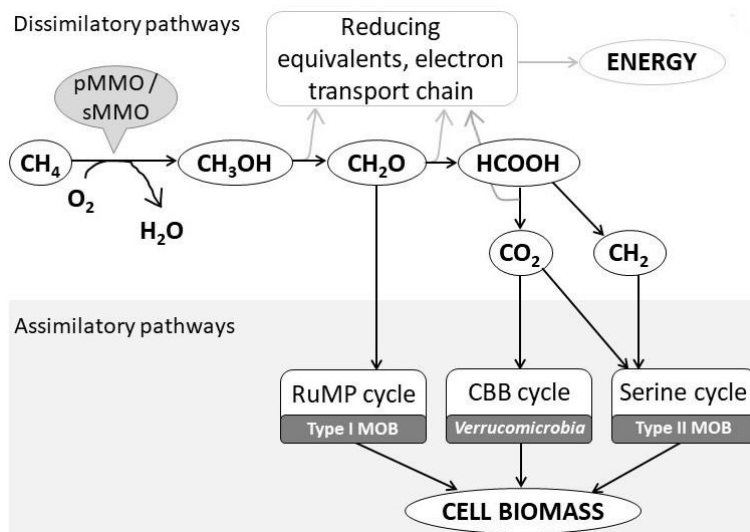
Figure 6. Metabolic pathways of methane oxidizing bacteria (MOB; pMMO/sMMO, particulate /soluble methane monooxygenase; RuMP, ribulose monophosphate; CBB, Calvin-Benson-Bassham) (adapted from Kalyuzhnaya et al., 2015; Khlemenina et al., 2018)

The expression of MMO enzymes and the occurrence and activity of different type of MOB is affected by prevalent environmental conditions, such as methane concentration, availability of copper and other nutrients and trace elements, temperature, and pH as discussed below. The enzyme pMMO has a high affinity for methane and the type I MOB are often the dominating type in environments with atmospheric (low) methane concentrations, while type II MOB dominates in the opposite environments (Hanson & Hanson, 1996; Semrau et al., 2010). Both types of MOB have been identified from landfill cover soils, where the methane supply varies depending on the age of the waste fill, efficiency of gas extraction system and season

(Ait-Benichou et al., 2009; Lee et al., 2018). The pMMO is present and active in the cells of MOB all the time, but sMMO is only synthesized and activated when the copper concentration of the growth environment is low ($\leq$5.6 µmol Cu g^{-1} protein; Semrau et al., 2010; Hakobyan & Liesack, 2020). In addition to copper, also other trace elements, such as iron and zinc, are require for synthesis of pMMO and sMMO (Semrau et al., 2010; Nikiema et al., 2013). Type I MOB are reported to have a higher tolerance to low temperatures (<10 °C) than type II MOB, owing to characteristics of their specific fatty acid composition, but in general, the low temperature decreases the activity of MOB significantly due to dominance of mesophilic vs. psychrophilic MOB in the community (Börjesson et al., 2004; Semrau et al., 2010; Islam et al., 2020). The temperature sensitivity of methanotrophs is evident especially in the boreal areas, where the activity of MOB decreases drastically during winter months resulting in a fall of the methane oxidation efficiency (for example from >96% to <22% in a Finnish landfill biocover) (Einola et al., 2008; Ait-Benichou et al., 2009; Lee et al., 2018). As for pH, the type I MOB have reported to have a slightly higher optimum pH range (6.7–8.2) than type II MOB (5.0–6.5) and, thus, the pH of the landfill cover soil affects partly the composition of MOB community (Su et al., 2014; Reddy et al., 2020).

MOB can be found from soil everywhere in the landfills, but there are great differences in the reported methane oxidation efficiencies, which can range from 20% to >96% of the methane emitted depending on the application and type of the top soil cover (Barlaz et al., 2004; Einola et al., 2008). Several different landfill soil materials and materials proposed to be used in landfill covers have been tested for their methane elimination capacities and removal efficiencies (Table 1). The greatest explanatory factor for the observed methane oxidation activities are the type and characteristics of the cover soil material (van Verseveld & Gebert, 2020). Organic, compost-based soils ($\geq$20 w-% organic matter) have found to support higher methane oxidation activities than mineral soils (<20 w-% organic matter; Toth et al., 2012) (Barlaz et al., 2004; Ait-Benichou et al., 2009; Lee et al., 2018). The use of spoils and different mineral soils in the final soil covers of the closed landfills, thus, can result in low methane oxidation activity and escape of methane to the atmosphere (Barlaz et al., 2004; Sadasivam & Reddy, 2014). The methane oxidation activity or the growth of the MOB, however, is not only attributed to the organic matter content but also to the other physical and chemical factors of the soil material (as reviewed below), which can be focused in planning and engineering the existing and new soil covers for supporting methane oxidation (Scheutz et al., 2009; Bajar et al., 2017).

Table 1. Methane elimination capacities of landfill cover and biocover materials determined by continuous column experiments (at 22±2 °C)

Material	Soil type	MC (% ww)	WHC (% dw)	OM (% dw)	Dry density (kg L^{-1})	Inlet load (g CH$_4$ m^{-2} d^{-1})	EC (g CH$_4$ m^{-2} d^{-1})	RE (%)	Ref
Landfill soil	Sandy loamy soil	16–25	31–41	3.6–5.2	1.26–1.35[a]	165	61–71	37–43	[1]
Landfill soil	Sandy loamy soil (60% sand, 32% silt, 8% clay)	14	n.r.	1.7 (carbon)	0.96–1.04	I. 160 II. 370	I. 152–159 II. 290	I. 95–99 II. 78	[2]
Landfill soil	Sand (6% gravel, 87% sand, 6% silt, 1% clay), no prior exposure to landfill gas	16.8[b]	n.r.	2.0	1.67	I. 39 II. 57 III. 80	I. 37 II. 46 III. 46	I. 95 II. 80 III. 58	[3]
Landfill soil	Loamy sand (4% gravel, 76% sand, 20% silt + clay), no prior exposure to landfill gas	31[b]	n.r.	7.5	1.36	I. 32 II. 53 III. 78	I. 13 II. 4 III. 14	I. 40 II. 7 III. 18	[3]
Amended landfill cover soil	25 : 4 : 71 silty clay soil (scs) : wood pellet char : scs (v-%)	5.8	19	0.8 : 96 : 0.8	1.43	I. 90 II. 77	I. 50 II. 36	I. 56 II. 47	[4]
Amended landfill cover soil	33 : 33 : 33 silty clay soil (scs) : scs with 2% wood pellet char and ash : scs (v-%)	6.4	21	0.8 : 2.8 : 0.8	1.47	I. 90 II. 77	I. 69 II. 39	I. 77 II. 51	[4]
Amended landfill cover soil	Intermediate cover soil, lean clay (v-%)	12.1	53	2.6	1.37	I. 90 II. 77	I. 50 II. 9	I. 56 II. 12	[4]
Compost biocover	Mixture of composted organic waste and municipal solid waste (1 year old)	30	n.r.	23–27	n.r.	I. 41 II. 206	I. 33 II. 41	I. 80 II. 20	[5]
Compost biocover	Mixture of composted organic waste and municipal solid waste (1 year old)	30	n.r.	23–27	n.r.	I. 82 II. 312	I. 77 II. 271	I. 94 II. 87	[5][c]
Compost biofilter	Composted yard and garden waste	49	71[d]	14	0.44	155	n.r.	100	[6]
Compost biofilter	Composted yard and garden waste, peat, spruce, fibers	46	77[d]	26	0.24	155	n.r.	65	[6]

MC, moisture content; WHC, water-holding capacity; OM, organic matter; ww, wet weight; dw, dry weight; EC, elimination capacity; RE, removal efficiency; n.r, not reported.

[a]The nature of density (bulk, dry) was not specified by the authors.   [b]Volumetric water content.   [c]Additional aeration from the bottom inlet of the column.   [d]Relative to moist mass.

[1] Huber-Humer et al., 2011; [2] De Visscher et al., 1999; [3] Rachor et al., 2011; [4] Yargicoglu & Reddy, 2017; [5] Pecorini & Iannelli, 2020; [6] Streese & Stegmann, 2003.

The most important characteristics of the top soil material are the physical structure of the material and moisture content, because the transport of the gaseous substrates (CH_4 and O_2) has to be optimal to enable maximal methane oxidation (Ait-Benichou et al., 2009; Chi et al., 2012). Flow of the gases by diffusion or advection in the soil is dependent on permeability of the material, which instead is governed by the number, size, spatial alignment and connectivity of the pores, and texture and density of the material (van Verseveld & Gebert, 2020). Depending on the moisture content of the material, some or all of the pores can be saturated, which disconnects the air-filled pores from each other, and the gas is transported by slower diffusion instead of faster advection (Ahoughalandari et al., 2017; van Verseveld & Gebert, 2020). The landfill cover soils undergo both dry and wet seasons resulting from varying weather conditions, so the partial saturation of the material pores is inevitable (Barlaz et al., 2004; Ait-Benichou et al., 2009). However, the gas permeability and tolerance of the soil material to irrigation can be affected by selecting materials with high water-holding capacity (WHC) such as compost (Barlaz et al., 2004), that can store a high amount of water relative to the total porosity enabling a higher share of pores to remain air-filled than a material with lower WHC (Reynolds et al., 2009). Thus, optimum moisture content of the soil material to support activity of MOB is approximately 30–50 w-% or 50–80% of the WHC (Huber-Humer et al., 2009; Christiansen et al., 2017).

Nitrogen and phosphorus are important macronutrients required by MOB (Hanson & Hanson, 1996). The nitrogen content of the soil material affects the composition of MOB community as type I MOB are thriving in environments with higher nutrient and nitrogen availability whereas type II MOB are more often encountered in environments with lower nitrogen availability due to their ability to fix N_2 (Lee et al., 2009; Semrau et al., 2011; Meyer-Dombard et al., 2020). The effect of nitrogen addition on the activity of MOB in soil has been studied by several authors and the effect seems to depend on the quantity and type of the nitrogen compound (for review, see Bodelier & Laanbroek, 2004). In most studies, NH_4^+ and NO_2^- addition inhibited and addition of NO_3^- enhanced the activity of MOB (e.g., Bodelier & Laanbroek, 2004; Zhang et al., 2011). However, opposite observations have also been reported (for example stimulation of methane oxidation activity by NH_4^+; Lee et al., 2009). As for phosphorus, the activity of MOB has been found to positively correlate with the ambient phosphorus content of the soil (Veraart et al., 2015), but the artificial fertilization with phosphorus has resulted in inconsistent responses of the MOB activity; in some cases the effect has been stimulating and in

some cases decreasing (Nikiema et al., 2010; Zhang et al., 2011; Jugnia et al., 2012; Zheng et al., 2013; Veraart et al., 2015). The role of phosphorus in governing the methane oxidation activity of the soils, however, has been suggested to be less significant than the effect of other soil features (pH, moisture content, availability of methane) (Su et al., 2014).

MOB do not directly benefit from the organic matter as a substrate, but the organic matter content of the landfill soil cover has several beneficial impacts on methane oxidation activity for example by increasing the porosity and WHC of the material (Huber-Humer et al., 2009; Lee et al., 2009). However, high organic matter content of the soil material can also activate heterotrophic bacteria responsible of basal respiration, which leads to competition over O_2 with MOB (Huber-Humer et al., 2009; Olorunfemi et al., 2016). A recommendation for the maximum activity of heterotrophic bacteria in the material used for supporting methane oxidation is <8 mg O_2 g^{-1} dry mass (measured over seven days; Huber-Humer et al., 2009). In addition to being competitive, the relationship of MOB with other microorganisms, such as autotrophic and heterotrophic bacteria, can be mutualistic and their co-existence has been reported to benefit the activity of MOB (Ho et al., 2014). The mechanism behind the mutualism has not been resolved (Stock et al., 2013; Ho et al., 2014), but one of the suggested explanations is that the heterotrophic bacteria consume the metabolic by-products of methane oxidation, such as methanol (Fig. 6), which could inhibit the activity of MOB if became accumulated (Hanson & Hanson, 1996; Murrell, 2010).

2.3.2   Sulfate reduction in deep geologic repositories

SRM are a vast group of anaerobic microorganisms comprising of different bacterial and archaeal species belonging to classes of δ-proteobacteria, Negativicutes, Clostridia, Thermodesulfobacteria and Euryarchaeota, among others (Muyzer & Stams, 2008; Rabus et al., 2015). All SRM use sulfate as a terminal electron acceptor and transform it to sulfide in dissimilative energy yielding metabolism but the electron donors and carbon sources are selected based on the autotrophy (CO_2, H_2) or heterotrophy (organic compounds) of the microorganism (Fig. 7; Liamleam & Annachhatre, 2007; Rabus et al., 2015). Part of the sulfate can be assimilated for synthesis of different cell constituents (e.g. proteins; Muyzer & Stams, 2008). In the lack of sulfate, SRM can also use other inorganic and organic compounds as terminal electron acceptors (e.g. sulfite, sulfur, nitrate, nitrite, iron(III); Muyzer & Stams,

2008). Majority of SRM prefer acetate and other fatty acids, lactate, and ethanol as electron donors, but some SRM can utilize also more complex hydrocarbons and organic matter (e.g. different organic wastes; Liamleam & Annachhatre, 2007; Kijjanapanich et al., 2014). As a result of sulfate reduction, the organic compounds can be oxidized either completely to CO_2 or incompletely to acetate (Fig. 7; Muyzer & Stams, 2008).
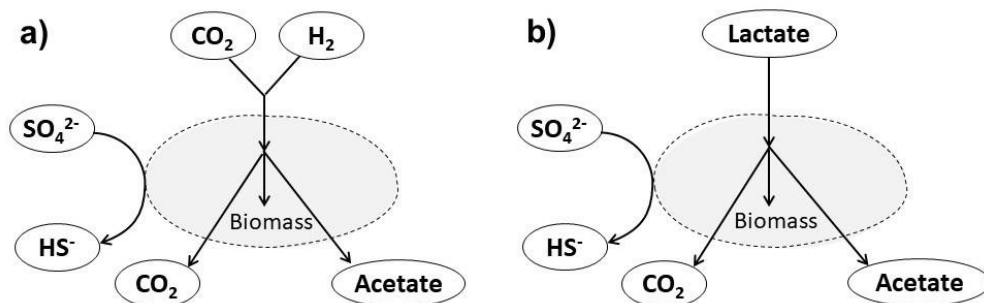
Figure 7. Examples of metabolic reactions by a) litoautotrophic and b) heterotrophic sulfate-reducing microorganism using sulfate as an electron acceptor (adapted from Liamleam & Annachhatre, 2007; Londry & Des Marais, 2013; Vita et al., 2015)

SRM are ubiquitous in nature and can be found from several different terrestrial and aquatic environments including the groundwater and bedrock of Olkiluoto and the bentonites planned for use in the EBS (Muyzer & Stams, 2008; Pedersen et al., 2014a, b; Bomberg et al., 2015). At the depth of 250–350 m in Olkiluoto bedrock, the brackish ($1 < TDS < 10$ g L^{-1}) sulfate-dominated and saline ($10 < TDS < 125$ g L^{-1}) chloride- and CH_4-dominated waters become mixed and, thus, the SRM have found to inhabit this depth range (Posiva, 2013; Pedersen et al., 2014a). Intrusion of the SRM-rich sulfate-dominated brackish water to the repository is possible through water-conducting fractures and following from construction-induced disturbances of the hydrogeochemistry of the site, creating a basis for sulfate-reducing activity also at the repository depth (420 m; Posiva, 2013; Pedersen et al., 2014a). In addition to groundwater, also the bentonites used as buffer and backfill materials contain naturally SRM. The number of indigenous SRM (<10 to 424 cells g^{-1}; Svensson et al., 2011; Stroes-Gascoyne et al., 2014; Grigoryan et al., 2018) and their diversity in different bentonites is affected by the origin of the bentonite and the different processes the batch undergoes after being excavated (e.g. drying, milling, transportation; Taborowski et al., 2019). The activity of SRM in the bentonite barriers is not desirable due to the threat of sulfide-induced copper corrosion, but it cannot be avoided due to natural existence of the microorganisms in the repository

environment (Pedersen et al., 2014a). The activity of SRM in the vicinity of the canister is attempted to be controlled artificially by engineering characteristics of the bentonite barriers but also the natural constraints are expected to limit the overall microbial and SRM activity in the repository environment as reviewed in the following (Table 2).

During early evolution of the repository, the activation of SRM in the unsaturated buffer is limited by low moisture content and high temperature (Svensson et al., 2011). Temperature emitting from the emplaced SNF canister dries the buffer and the water vapor condenses on the cooler areas of the bentonite and host rock (King et al., 2017). Even though most of the SRM species are mesophilic, they include thermophilic species being able to grow at temperatures as high as 110 °C, and they can survive extended periods of high temperature through formation of endospores, which can become vegetative cells when the conditions turn more favorable (Karnland et al., 2009; Stroes-Gascoyne & Hamon, 2010; Rabus et al., 2015). The activity of SRM is expected to be limited in the buffer also after the decay heat of the SNF decreases and the groundwater starts entering the buffer as saturated vapor because the SRM require aqueous water and a moisture content of ≥ 15 weight (wt)-% for survival and growth (Table 2; Stroes-Gascoyne et al., 1997; Svensson et al., 2020). Saturation of the bentonite leads to build-up of the swelling pressure in the buffer installed at initial average dry density of 1595 kg m^{-3} (Section 2.2.2; Juvankoski, 2013), which again is designed to inhibit the activity of SRM and other microorganisms indigenous or those transported to the buffer along with groundwater (Pedersen, 2002). The sensitivity of SRM activity to bentonite densities (1420–2010 kg$_{wet}$ m^{-3}) has been tested with several different Na- and Ca-bentonites inoculated with SRM and the density range where the activity of SRM dropped from detectable to below limit of detection varied between different bentonites (Table 2; Taborowski et al., 2019).

Table 2. Sulfate reduction activity associated with bentonites in varying conditions relevant for disposal of spent nuclear fuel

Studied factor affecting microbial activity/viability	Scale	Experimental settings	Microorganisms	Main findings	Ref.
Water availability in bentonite	Batch: gas-tight tubes and larger containers, duration 3 months–1 year	Wyoming Na-bentonite (MX80), liquid, and gaseous water (RH 11, 76, 97 and 100%), lactate and bentonite OM as a substrate	*Pseudodesulfovibrio aespoeensis*, groundwater microorganisms, unsterilized bentonite	SRM activity only in the presence of liquid water, not at RHs 11–100%. Activity only in tests with lactate, not with bentonite OM. Indigenous bentonite microorganisms activated.	[1]
Water availability in bentonite	Batch: RH controlled aerobic chambers, duration 1 year	Wyoming Na-bentonite (MX80), inoculated and desiccated bentonite films, RH 30 and 75%, added substrates or bentonite OM	Enriched bentonite microbial community	Higher RH (75%) sustains the activity of aerobic microorganisms longer than lower RH (30%), but the viability is higher after incubation at lower RH. Bentonite OM induced activity but to lower extent than added substrates.	[2]
Moisture content of bentonite	*In situ:* depth of 240 m in granitic rock (Canadian shield), duration 2.5 years	Avonlea Na-bentonite + sand, ρ_d ~1700 kg m^{-3}, deposition hole ⌀1.24 m x 6 m (embedded thermal element 0.64 x 2.25 m), moisture content 19.5–23%	Natural microbial community; aerobic and anaerobic microorganisms, fungi	No activity of SRM during the experiment due to oxic conditions. Post-experiment viability of SRM detected from samples having moisture content ≥15 wt-% translating to water activity of >0.96.	[3]
Density, porewater salinity, SP and water availability of bentonite	Cell: compacted bentonites (4–5 mL) saturated with different solutions, duration 40–90 d	Wyoming Na-bentonite (MX80), ρ_d 800, 1300, 1600, 1800 and 2000 kg m^{-3}, saturation solution salinities 0, 50, 100, 150 and 200 g NaCl L^{-1}	Indigenous bentonite microorganisms; aerobic heterotrophs	The bacteria remained culturable in samples having water activity >0.96, SP <2 MPa and ρ_d <1600 kg m^{-3}. Water activity, density and SP were affected by pore water salinity; at salinity ≤50 g L^{-1}, ρ_d of ≥1600 kg m^{-3} is required to obtain water activity <0.96 and SP >2 MP	[4]
Density	Cell: compacted bentonites (20 mL) saturated with saline solution, duration 35–123 d	Wolclay MX80 (Na), Indian Ashapura (Na), German Calcigel (Ca) and Czech Rokle (n.a.), ρ_w 1500–2000 kg m^{-3}, lactate and bentonite OM as a substrate	*Pseudodesulfovibrio aespoeensis*, *Desulfotomaculum nitrificans*, *Desulfosporosinus orientis*, unsterile bentonite	Activity of SRM decreased from detectable to below detection at varying densities for different bentonites: e.g. 1750–1847 kg m^{-3} for MX80 and 1687–1795 kg m^{-3} for Ashapura. The production of sulfide ceased at SP >1 MPa.	[5]

RH, relative humidity; OM, organic matter; SRM, sulfate-reducing microorganisms; SP, swelling pressure; ρ_d ρ_w, dry, wet density; n.a., not applicable.

[1] Svensson et al., 2020; [2] Stone et al., 2016; [3] Stroes-Gascoyne et al., 2010; [4] Stroes-Gascoyne et al., 1997; [5] Bengtsson & Pedersen, 2017; Taborowski et al., 2019.

The control of microbial activity by high density ($\geq$1400 kg m^{-3}) at full saturation is attributed to the resulting high swelling pressure ($\geq$2 MPa), low amount of free water (water activity $\leq$0.96) and small pore size of the bentonite (Stroes-Gascoyne et al., 2010; Kaufhold et al., 2015). Microorganisms can resist the osmotic pressure in bentonite by creating turgor pressure inside the cells (Koch, 2001) and for example certain species of SRM, but not all, can grow at pressures as high as 40 MPa (Khelaifia et al., 2011). However, the amount of free water in the aggregate voids of the bentonite (Fig. 5) has a great effect on the activity of SRM and other microorganisms, whose activity stops at or below water activity of 0.96 obtained at swelling pressure of $\geq$6 MPa (Motamedi et al., 1996; Stroes-Gascoyne et al., 1997, 2010). Moreover, the pore diameter of for example Wyoming type bentonite at dry density of 1630 kg m^{-3} is approximately to 0.005–0.1 μm (at 0–100 g TDS L^{-1}; Stroes-Gascoyne et al., 2010), which is less than the size of a SRM (0.4–3.0 μm; Kushkevych, 2020). Some activity of spore forming SRM has been detected in sediment samples having pore diameter of 0.01–0.03 μm, but on average the subsurface anaerobic bacteria require pore throats of size >0.2 μm to be able to maintain metabolic activity and viability (Fredrickson et al., 1997). The combined effect of time and high pressure also affects the survival of the SRM as, for example, the endospores of SRM trapped inside the dense bentonite have not shown to regain their viability in improved conditions after eight years of dormancy inside compacted bentonite ($\geq$1600 kg$_{dry}$ m^{-3}, swelling pressure 2 MPa; Jalique et al., 2016).

Even though the density inhibits microbial activity inside the bulk bentonite, microorganisms can be active at the interfaces of the bentonites and host rock where the density might be lower and, consequently, the porosity and water activity higher than in the bulk bentonite (Stroes-Gascoyne et al., 2014). The amount (<40 mg L^{-1}) and type of organic compounds available for the microorganisms in the groundwater environment is poor, but a concern has emerged for the bentonite natural organic matter introducing an additional source of organic compounds in the repository for microbial consumption (Hallbeck, 2010; Posiva, 2012b; Marshall & Simpson, 2014). One factor possibly limiting the release of organic matter from bentonite is transport kinetics, which are affected by the size and location of the compound in the bentonite (Durce et al., 2015, 2018; Zhu et al., 2016). Majority of the organic matter is adsorbed on the surfaces of the montmorillonite particles, thus, contributing to the stable pool of organic matter in the bentonites, while part of the organic matter can be mobilized by leaching or extraction (Durce et al., 2015; Zhu et al., 2016). The mobile fraction is also available to microbial degradation (Zhu et al., 2016). The

maximum allowed concentration of organic matter in the bentonite used for the buffer is 10 000 mg kg^{-1} (Juvankoski, 2013) but only ≤8% of this has been suggested to be water-extractable and the remaining being immobile (Marshall et al., 2015).

Some studies have suggested that bentonite organic matter in general is not a suitable substrate for the SRM (e.g. Svensson et al., 2020), while in other studies, the consumption of bentonite organic matter has been the only plausible explanation for activity of SRM in the bentonite (Table 2; Stone et al., 2016; Bengtsson et al., 2017). The bentonite organic matter consists mostly of long-chained aliphatic compounds, which are large in size (in Boom Clay, 60–80% of organic matter is 50 kDa–0.45 μm) and chemically recalcitrant (Durce et al., 2015; Marshall et al., 2015), but also some low-molecular-weight compounds have been identified (Taborowski et al., 2019). In general, the SRM prefer low-molecular-weight compounds, such as H$_2$, over the more complex compounds as electron donors because the free energy change is higher from oxidation of simpler than more complex compounds (Liamleam & Annacchatre, 2007). In addition, the SRM do not oxidize the hydrocarbons completely (Liamleam & Annacchatre, 2007). However, the SRM can benefit from the activity of fermentative and hydrolytic microorganisms, which degrade the hydrocarbons to smaller compounds, which are more utilizable by the SRM (Muyzer & Stams, 2008; Zavarzin et al., 2008). The fermentation and methanogenesis along with sulfate reduction are the dominant metabolic types at the deep depths (300–800 m) of Olkiluoto bedrock (Bomberg et al., 2016).

The macronutrient requirement of the SRM is rather low, 100 : 5 : 1 organic carbon : N : P (organic carbon determined as theoretical oxygen demand, the ratio in optimized laboratory conditions), but their growth is believed to be limited by the availability of nitrogen and phosphorus in the repository environment (Stroes-Gascoyne, 1989; El Bayoumy et al 1999; Marshall & Simpson, 2014). The nutrient availability is the groundwater environment is low currently, for example in Olkiluoto groundwater <0.02 mg NH$_4^+$ L^{-1} and <0.03 mg PO$_4^{3-}$ L^{-1}, and it is expected to remain low also in the future as the glacial melting waters infiltrating to the repository depth are expected to have a low overall nutrient content (Hellä et al., 2014). Construction of the repository introduces a small amount of nitrogen compounds to the repository via use of explosives and the bentonites used in the EBS contain some nitrogen and phosphorus in their accessory minerals (<0.05 w-% N, <0.25 w-% P$_2$O$_5$) but they are not believed to increase the nutrient inventory of the repository environment significantly (Stroes-Gascoyne, 1989; Marshall & Simpson, 2014; Kiviranta et al., 2018). The most important nutrient for the activity of SRM is sulfate (Kijjanapanich et al. 2014), availability of which is generously

contributed by the sulfate in the groundwater (currently 460 mg L^{-1} in the brackish and 20 mg L^{-1} in the saline water) and gypsum dissolving from the bentonites (for example 9000 mg kg^{-1} in a Wyoming type Na-bentonite) (Juvankoski, 2013; Hellä et al., 2014; Wersin et al., 2014).

3 RESEARCH OBJECTIVES

The main objective of this thesis was to assess the effect of different soil and bentonite materials used or planned to be used in the engineered barriers of subterranean waste disposal on selected microbial processes. Moreover, the objective was to identify factors by which the microbial processes could be managed. The main objective can be divided into four sub-objectives of which the first two are related to landfilling of MSW and the two latter to disposal of SNF (the number of paper/-s addressing the objective is shown in the parentheses):

- To characterize the activity of methane-oxidizing bacteria in soils of different landfill cover structures and to identify the soil characteristics governing their activities (I)

- To assess different methods for managing methane oxidation activity in aged landfill soil aiming at enhancing mitigation of methane emissions from the landfills (I, II)

- To assess the role of bentonite organic matter of different bentonites as a substrate of sulfate-reducing and other microorganisms representative of those in SNF repository environment (III)

- To assess how simulated repository conditions affect the quantity and release rate of soluble organic matter from bentonites, which are examples of materials that could be used for disposal of SNF (III, IV)

4 MATERIALS AND METHODS

4.1 Experimental design

An overview of the objectives and experiments conducted in this thesis is presented in Table 3. Soil and compost-based samples collected from two landfills having different types of cover structures were characterized for the chemical characteristics and activity of MOB by batch assays. Based on the results from the characterization, two landfill cover samples were selected to assess the effect of different methods on enhancing the activity of MOB in batch assays and a continuous column experiment. The growth of the SRM and other microorganisms on bentonite organic matter was assessed in a semi-continuous cell experiment and in post-experimental batch assays. The effect of the microbial activity and other simulated repository conditions (e.g. compaction to high dry density) on bentonite soluble organic matter was assessed in post-experimental dynamic leaching assays.

Table 3. Summary of the experiments conducted in this thesis

Objective	Engineered barrier material	Monitored micro-bial processes	Experiments	Paper
To characterize methane oxidation activity and identify the governing factors	Landfill cover soils	Methane oxidation, basal respiration	Chemical analyses, batch assay	I
To improve methane oxidation activity by fertilization, decompaction of soil and compost addition	Landfill cover soils	Methane oxidation	Batch assay, continuous column	I, II
To assess the growth of microorganisms on bentonite organic matter	Bentonite	Sulfate reduction, methane production, iron reduction	Semi-continuous cell, batch assay	III, IV
To assess the effect of bentonite compaction and microbial activity on bentonite organic matter	Bentonite	Sulfate reduction, methane production, iron reduction	Cell, dynamic leaching assay	IV

4.2　Methane oxidation in landfill cover soils

4.2.1　Landfill cover soils

The soil and compost-based samples were collected from different locations of Ämmässuo (in operation, six samples) and Tarastenjärvi (in a post-closure phase, six samples) landfills, Finland, both of which had received municipal, construction and industrial wastes (Fig. 8). The sampled biocovers of Ämmässuo had been distributed five (2007; As1 and As2) and two years (2010; As3) before sampling and they consisted of mixture of compost, clay, and sand. The samples were collected from two depths (10–20 and 40–50 cm) of the approximately 50 cm deep biocovers (Fig. 8; paper I). The sampled cover soils of Tarastenjärvi landfill represented different soil types; six years old final cover soil (Tj1 and Tj2) and sand (Tj3), approximately 20 years old final cover soil from locations emitting (Tj4) and not emitting methane (Tj5) and one year old compost-based mixture from freshly landscaped area (Tj6; paper I). From Tarastenjärvi, the samples were collected from one pooled depth (5–20 cm). The soil material collected from each sampling location (5 L) was stored at 4 °C prior to chemical and microbiological (MOB activity) characterization, and batch and column experiments to assess the effect of different methods on MOB activity (Fig. 8; Sections 4.2.3–4.2.4).
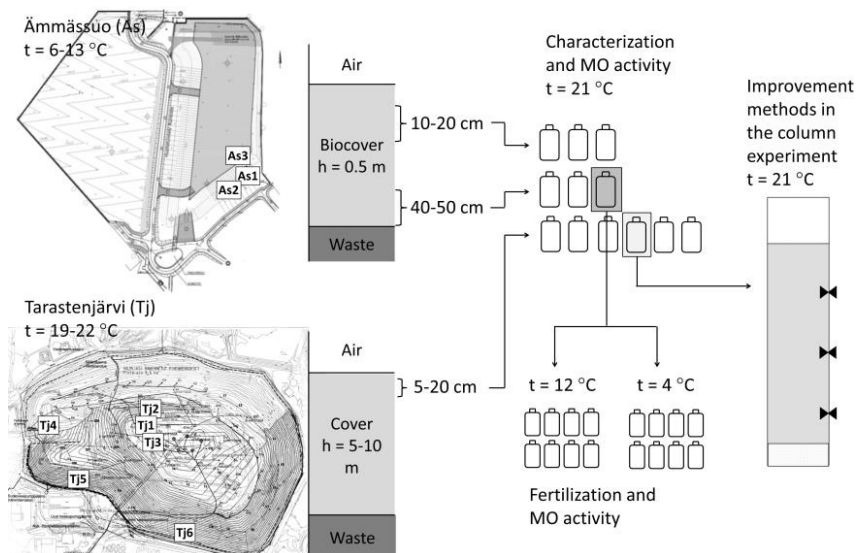
Figure 8.　The sampling locations in Ämmässuo (As1–3) and Tarastenjärvi (Tj1–6) landfills and the experimental design (MO, methane oxidation; adapted from papers I and II)

4.2.2   A batch assay for measuring MOB activity and basal respiration

The activity of MOB in different landfill cover soil samples was characterized by measuring the methane oxidation rates (MORs) in batch assays. In the assay, sub-samples (8–12 g) of each sieved moist soil material (<2 mm; As1–As3, Tj1–Tj6; $n = 2$) were inserted in gas-tight glass bottles, where also methane was added through the cap at final concentration of 7 v-% (Aga Ltd., Finland). Samples for measuring the basal respiration of heterotrophic soil bacteria ($n = 2$) were prepared similarly but without methane addition. The bottles were incubated at 21±2 °C and the concentrations of methane and CO_2 were measured from the gas phase regularly until methane had been used up by the bacteria (<0.1 v-%; 23–360 h depending on the sample). To determine the factors governing the MOB activities in the landfill soil materials, the soils were characterized for pH, electrical conductivity and moisture, organic matter, nitrate and phosphate contents before the batch assay.

The samples collected from Ämmässuo landfill were stored for five weeks prior to the assay due to method optimization and preliminary testing. Therefore, they were pre-incubated with methane to induce the activity of MOB to a similar level expected to occur in samples of Tarastenjärvi, which were analyzed shortly after collecting from the landfill (0–4 d, no preliminary incubation). The moisture content of the samples Tj1–3 and Tj5 from Tarastenjärvi was increased with distilled water (to 33% wet weight, corresponding to approximately 50% of the average WHC, considered optimal for the MOB; Huber-Humer et al., 2009) for the assay as their original moisture content was not high enough to support the activity of MOB based on initial testing (paper I). The moisture content of the Ämmässuo samples was not adjusted (originally 31% wet weight on average).

4.2.3   Effect of nutrient additions on MOB activity

The effect of nutrient addition on MOB activity was tested in a batch assay by using the compost sample from Ämmässuo (As2 40-50) because it contained less nitrate and phosphate and exhibited a lower MOB activity than the other samples (see Section 5.1.1 for results). The batch assay was conducted at two temperatures, 4 °C and 12 °C, because maintaining the activity of the MOB at low temperature is critical for emission control. The studied nutrients included nitrogen (N), phosphorus (P) and trace elements (TE) in solutions prepared as follows per liter of distilled water: solution N: 31.1 g KNO_3, 24.7 g $MgSO_4 \cdot 7H_2O$, 4.6 g $CaCl_2$; solution P: 14.6 g Na_2HPO_4, 26.8 g KH_2PO_4; solution TE: 4.7 mg $CuSO_4 \cdot 5H_2O$, 11.5 mg $FeSO_4 \cdot$

$7H_2O$, 9.2 mg $ZnSO_4 \cdot 7H_2O$, 6.3 mg $EDTA\text{-}Na_2 \cdot 2H_2O$, 0.45 mg $MnCl_2 \cdot 4H_2O$, 0.30 mg $NiSO_4 \cdot 6H_2O$, 5.9 mg $Na_2MoO_4 \cdot 2H_2O$. The assay was created as a full factorial, balanced, and randomized design with one level of three factors. Thus, the samples were treated with a combination of one (N, P, TE), two (N+P, N+TE, P+TE) or three (N+P+TE) nutrients.

The batch assay was conducted in gas-tight bottles at 4 °C and 12 °C as described earlier (Section 4.2.2), using 10–12 g wet weight of sample (As2 40–50) and the nutrients were added to the samples by spraying (270 µL) (paper I). The nutrient additions resulted in concentration of 650 mg NO_3^- kg^{-1}, 960 mg PO_4^{3-} kg^{-1} and 0.040 mg Cu^{2+} kg^{-1} (including both the initial and added concentrations of the nutrients), which were selected based on the nutrient contents reported to stimulate the activity of MOB (Wang et al., 2011; Nikiema et al., 2013; van der Ha et al., 2013).

4.2.4 Effect of improvement methods on MOB activity

The effect of fertilization, decompaction and amelioration with compost on MOB activity in the landfill soil was assessed with a continuous column experiment. The soil sample from Tarastenjärvi (Tj4) was used in the experiments because it exhibited the highest MOB activity of all the studied mineral soil and compost-based samples collected from Tarastenjärvi and Ämmässuo landfills even though its nutrient, organic matter and moisture contents were lower than in many of the studied compost-based materials (for results and discussion, see Section 5.1.1). Thus, this soil was expected to contain an active community of MOB, whose response to manipulation of the initially low nutrient and organic matter contents with fertilization and compost amelioration could be observed. For the column experiment, a fresh batch of soil was obtained from Tarastenjärvi and sieved to ensure homogeneity of the material (size fraction of <19 mm; 39.8% gravel, 49.9% sand and 10.3% silt). As the soil could be classified as well-graded silty gravelly sand (Whitlow, 2001) or mineral soil (<20 w-% organic matter; Toth et al., 2008), it was expected to be prone of compaction and pore clogging. Therefore, decompaction could restore the porosity of the soil and compost addition to improve its air capacity (and increase the nutrient and organic matter content). The moisture content of the soil was increased to 34% wet weight with distilled water, which equaled 50% of the WHC (Huber-Humer et al., 2009).

The experiment was carried out in two continuously gas fed acrylic columns at 21±2 °C (60 × 14 cm, height × diameter; Fig. 9; paper II). Approximately 9.3 kg

moist soil was placed inside the columns at bulk density of 1.3 kg L^{-1}, to match with the typical bulk density of mineral landfill cover soils (1.24–1.35 kg L^{-1}; Huber-Humer et al., 2011). The operation of a landfill cover was simulated by introducing continuously synthetic landfill gas (CH$_4$: CO$_2$, 50 : 50 v-%; Aga Ltd) in the column through the bottom inlet and pressurized air above the soil bed through the side inlet (Fig. 9). The used load of synthetic landfill gas relative to the surface area of the soil was 110 g CH$_4$ m^{-2} d^{-1} (days 0–24) and 210 g CH$_4$ m^{-2} d^{-1} (days 25–148). The used methane loads matched with the typical loads reported for different MSW landfills (21.5–518 g CH$_4$ m^{-2} d^{-1}; Einola et al., 2008; Reddy et al., 2014).
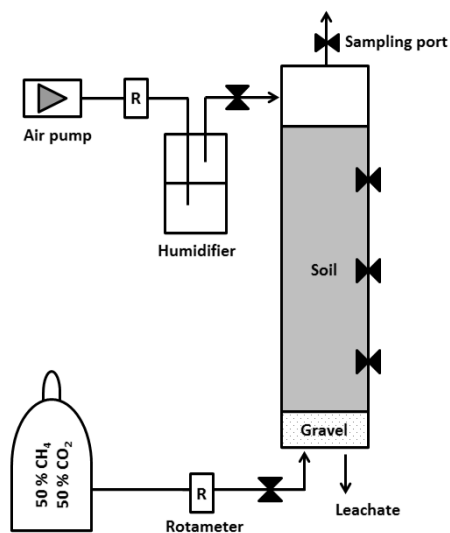
Figure 9. Schematic of a column setup used for simulating performance of a landfill cover (paper II)

The two columns were operated first similarly, and then different improvement methods were tested in one column (referred to as treatment column), while the other column was operated as a control column (paper II). The methods were applied to the treatment column on four occasions: fertilization (days 64–79 and again at days 113–114 because the first fertilization seemed ineffective), decompaction of the soil bed by mixing (day 97) and amelioration of soil with compost (day 120). As for the control column, distilled water was added instead of fertilizers (days 64–79 and 113–114) and the soil bed was decompacted without addition of compost to monitor the possible effect of increased moisture content of the soil upon fertilization and mechanical disturbance of the soil during compost amelioration on the methane elimination capacity (days 97 and 120) (paper II). The

soil was sampled for determination of chemical characteristics and MOB activity (by a batch assay as described in Section 4.2.2) prior to applying each method.

In fertilization (days 64–79 and 113–114) the nutrient solutions were injected to the soil through the top and side ports of the treatment column (Fig. 9). In the first fertilization, the added nutrient levels were 150 mg PO_4^{3-}, 400 mg NO_3^-, 500 mg SO_4^{2-} and 0.385 mg Cu^{2+} per kilogram of wet soil in the column, while in the second fertilization, they were 400 mg PO_4^{3-}, 600 mg NO_3^- and 750 mg SO_4^{2-} per kilogram of wet soil (paper II). The concentrations were selected based on values reported to fulfill the requirements of the MOB (Huber-Humer et al., 2009; van der Ha et al., 2010). In decompaction (day 97), soil was removed from both columns in three layers (0–10, 10–27 and 27–45 cm) based on orange-red coloration concentrated in the middle layer of soil, which suggested enrichment of MOB (Humer & Lechner, 1999). The soil was sampled for chemical and microbiological characterization, mixed mechanically to homogenize the soil structure, and inserted back to the columns at initial height (45 cm). In compost addition (day 120), the soil was removed from the treatment column and mixed with freshly obtained compost (particle size <20 mm; woodchips and sewage sludge) in dry weight ratio of approximately 80:20 soil : compost, a ratio considered feasible in practice in a real landfill. The mixture was re-packed into the column to the initial height (45 cm).

4.3 Sulfate reduction in simulated repository conditions

4.3.1 Bentonites

The three bentonites used in the experiments were originating from Wyoming, Indian and Bulgaria (Table 4). The bentonites from Wyoming and India had sodium as the main exchangeable cation while the bentonite from Bulgaria had calcium as the main exchangeable cation (Table 4). For the cell experiment (Section 4.3.2), the original Indian and Bulgarian bentonites were ground from grain size of 0.5–3 mm to <0.2 mm to ensure homogeneity of the material, while the Wyoming bentonite was used as is because of the finer original grain size (68% ≤0.5 mm; Kiviranta & Kumpulainen, 2011; Table 4). After arrival to the laboratory, the bentonites were stored in sealed containers at 6 °C for 24 months prior to the experiments (papers III and IV). Their former storage conditions were not known.

Table 4. Mineral composition and major elements as oxides of Wyoming[a], Indian[b] and Bulgarian[c] bentonites (mass-% of the dry material) (adapted from papers III and IV)

		Wyoming	Indian	Bulgarian	
Minerals	Smectite	88	74	68	*Wyoming bentonite*
	Illite	<1	1	7	
	Calcite	<1	9	11	
	Gypsum	<1	1	<1	
	Pyrite	1	0	0	
	Other	11	15	14	
					Indian bentonite
Elements	Sulfate-S	0.12	0.06	0.05	
	Sulfide-S	0.15	<0.02	<0.02	
	Fe^{3+}	2.1	10.6	3.2	
	Fe^{2+}	0.57	0.05	0.26	
	Inorganic C	0.15	0.36	n.r.	
	Organic C[d]	0.20	0.07	<0.14	
	LOI	6.3	12	17	*Bulgarian bentonite*
	CEC	930	880	630	
	Exchangeable cations (equivalent g^{-1})				
	Na	580	450	60	
	K	20	0	20	
	Ca	240	270	450	
	Mg	90	160	100	

n.r., not reported; LOI, loss on ignition; CEC, cation exchange capacity by NH_4Cl method (equivalent g^{-1}).

[a]Kiviranta & Kumpulainen, 2011; Kiviranta et al., 2018; [b]Kumpulainen & Kiviranta, 2015; [c]Kumpulainen et al., 2016. [d]Determined in this work.

4.3.2 Simulation of repository conditions in a cell experiment

To study the growth of SRM and other microorganisms on bentonite organic matter in simulated repository conditions, the experiment was set up with in total six parallel semi-continuous cells designed for this purpose (Fig. 10; paper III). Two cells were prepared for each of the three studied bentonites (Wyoming, Indian and Bulgarian; Table 4). A scenario of active microbial community at the interface of bentonite and bedrock was mimicked with one of the two cells, referred to as 'inoculated cells'. The sand layer of the other cell of each bentonite was not inoculated with microorganism and with these cells, referred to as 'uninoculated cells', a possible scenario of propagation of microorganisms indigenous to unsterilized bentonites was mimicked.
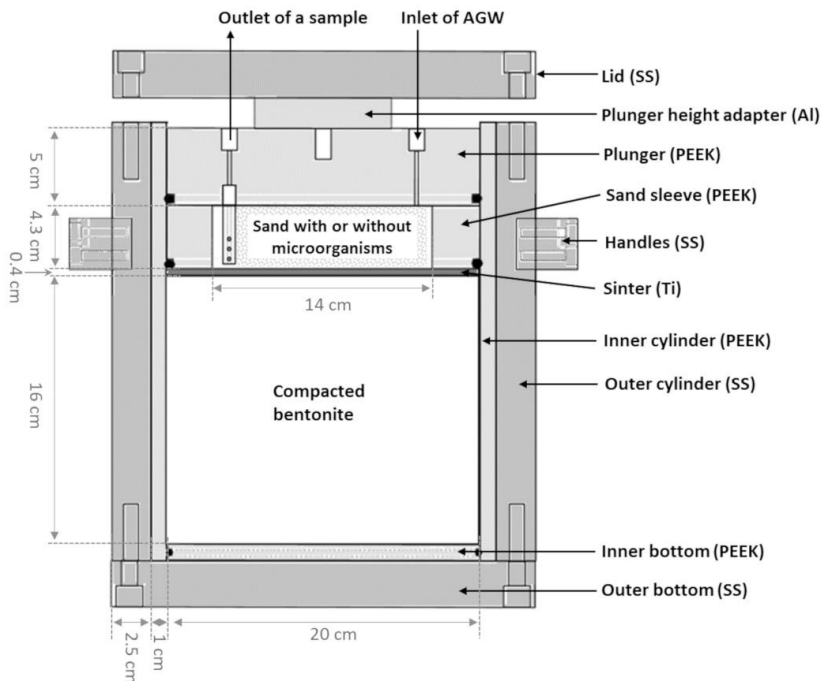
Figure 10. Schematic of the experimental cell (AGW, saline artificial groundwater; SS, stainless steel; PEEK, polyether ether ketone) (paper III)

For setting up the cell systems, the three studied bentonites (Wyoming as received, Indian and Bulgarian as ground; 7.0 kg dry weight) were degassed with N_2 (99.5 v-%, Aga Ltd.) and mixed with saline artificial groundwater (AGW) to saturate the pores of the bentonite upon compaction. The composition of saline AGW mimicked the groundwater in Olkiluoto at the repository depth (6.65 g NaCl, 4.77 g $CaCl_2 \cdot 2H_2O$, 519 mg $MgCl_2 \cdot 6H_2O$, 56.7 mg NaBr, 42.6 mg $SrCl_2 \cdot 6H_2O$, 29.6 mg Na_2SO_4, 21.0 mg KCl, 7.4 mg H_3BO_3, 2.2 mg NaF, and 0.03 mg NH_4Cl in 1 L distilled water; modified from Hellä et al., 2014). After mixing with saline AGW, the bentonites were compacted to a target dry density of 1400 kg m^{-3} (volume approximately 5 L) to inhibit the activity of microorganisms within the bentonite blocks (Taborowski et al., 2019). Due to technical reasons before start up and during the experiment, the bentonite blocks swelled, and they had to be recompacted. The dry densities remained at 1314–1368 kg m^{-3} (from operational day 167 onwards) as the initial target dry density (1400 kg m^{-3}) could not be restored (papers III and IV). After compaction of the bentonite blocks, a titanium sinter (pore size 1–2 μm) was placed on top of the bentonite and a layer of quartz sand ($0.63 \leq x < 1$ mm) saturated with saline AGW was assembled on top of the sinter (Fig. 10) to mimic the interface

of bentonite and bedrock (paper III). The sand layer of the inoculated cells was inoculated with SRM (*Desulfobacula phenolica*, *Desulfobulbus mediterraneus*, *Desulfobulbus rhabdoformis*, *Pseudodesulfovibrio aespoeensis*, and *Desulfotomaculum acetoxidans* from DSMZ GmbH) and microorganisms enriched from Olkiluoto groundwater (paper III). No microorganisms were added to the uninoculated cells. After setting up the sand layers, the cells were closed and sealed to maintain anaerobic conditions, and the experiment was started.

During the experiment, sub-samples were collected from the sand layer solution through the sampling valves (Fig. 10) every three weeks and analyzed for redox potential (E_h vs. standard hydrogen electrode) and concentrations of dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), sulfate, total iron, and methane (paper III). The cells were operated for 370–454 days and at the end of the experiment, the cells were opened and sand layers sampled for determination of elemental composition of the precipitates in the sand grains, activity of overall microbial community (by adenosine triphosphate [ATP] concentration) and activity of SRM by batch assays (paper III). The bentonite blocks were divided into layers depth-wise (6–7 layers) and sampled for determination of number of SRM (only the surface layer below sinter; paper III) and dry density and soluble organic matter (sOM) content (paper IV).

4.3.3   Post-experimental batch and dynamic leaching assays

The activity of SRM was determined in a batch assay by measuring the sulfate reduction rates (SRRs) of the SRM in the sand collected from the cells after the cell experiment (paper III). In the assay, samples (10 g) of moist sand from each of the six cells ($n = 3$) were inserted in gas-tight glass bottles containing N_2 gas phase and 140 mL of degassed growth medium (680 mg SO_4^{2-}, 500 mg Na-lactate, 50 mg yeast extract, 50 mg K_2HPO_4, 100 mg NH_4Cl, and 55 mg $NaHCO_3$ per liter of water; modified from DSMZ, 2017). The sand samples were incubated on a mixer at 200 rpm and 30 °C and samples were collected from the medium for determination of sulfate concentration over 8–75 days. One abiotic control sample was prepared as described above, but it was sterilized (60 min at 121 °C) before incubation.

The sOM contents of the three original bentonites (as received) and the bentonites from the six cells (divided into 6–7 horizontal layers) after the cell experiment were determined by dynamic leaching assays as follows. Bentonites (0.5–0.75 g dry weight) were mixed with 40 mL of leachant ($n = 3$–4 for each bentonite).

The leachant consisted of dilute AGW containing per liter of distilled water: 650 mg NaCl, 200 mg $CaCl_2 \cdot 2H_2O$, 150 mg $MgCl_2 \cdot 6H_2O$, 1.8 mg NaBr, 1.5 mg $SrCl_2 \cdot 6H_2O$, 135 mg Na_2SO_4, 18 mg KCl, 1.7 mg H_3BO_3, 1.3 mg NaF, and 2.2 mg NH_4Cl (modified from Hellä et al., 2014). Dilute AGW was selected as the leachant based on preliminary testing (paper IV). The bentonite samples were leached on a mixer (170 rpm) at $21\pm2\,°C$ for minimum of 24 h after which the bentonites were separated from the leachant by centrifugation (30 min at 10 000 x g). The separated leachant was analyzed for DOC concentration as a measure of sOM. Next, fresh batches of leachant were mixed with the separated bentonite samples. The leaching and leachant replacement steps were repeated until no more organic matter was released from the bentonite samples (4–24 times depending on the sample). In addition to samples containing bentonite, analytical blank samples were prepared from the leachant without bentonite and treated similarly as described above. The cumulative liquid-to-solid (L/S) ratios and sOM contents were calculated based on the volume of leachant used and the amount of DOC measured in the individual steps of the assay. For some of the samples, the leaching was stopped prematurely before the release of sOM had stopped due to time constraints (paper IV); Wyoming bentonite from inoculated (depths 4–16 cm) and uninoculated cell (1–6 and 12–16 cm) and original Bulgarian bentonite and from uninoculated cell (depths 1–16 cm). Thus, the sOM quantities reported for these samples were likely underestimating their actual sOM contents (paper IV).

4.4   Analytical and statistical methods and calculations

The analytical methods used in this thesis are summarized in Table 5.

The concentration of different analytes in liquid (e.g. DOC, anions) or gaseous samples (e.g. methane) are represented relative to volume of the sample. The contents of different analytes in solid samples (e.g. sOM, nutrients) are represented relative to the dry weight of the sample unless otherwise stated (e.g. moisture content relative to wet weight).

The MORs and SRRs were calculated for determining activities of MOB and SRM (Sections 4.2.2, 4.3.3). MORs were calculated from the linear part of the slope of methane concentration decreasing over time. Basal respiration was calculated from the increase in CO_2 concentration over time in samples not incubated with methane while the amount of CO_2 produced by the MOB was calculated by subtracting the amount of CO_2 produced by basal respiration from the amount of

CO$_2$ produced in the samples incubated with methane (papers I, II). SRRs were calculated from the linear part of the slope of sulfate concentration decreasing over time (paper III). The rates are presented relative to the dry weight of the sample.

Table 5.   Analytical methods used in the experiments

Analysis	Method	Paper
TS/moisture content and OM (as volatile solids) (s)	SFS-EN 14346, SFS-EN 15169 APHA, 1995	I–II III–IV
TOC (s)	CSN ISO 10694, CSN EN 13137 (by ALS Finland Oy)	IV
WHC (s)	Gravimetric method (FCQAO, 2003)	II
Elemental analysis (s)	SEM/EDS (Jeol JSM-IT-500) (focus on Fe and S)	III
Cu^{2+} (s)	Atomic absorption spectroscopy (Quevauviller, 1998)	II
Anions (NO$_3^-$, PO$_3^{4-}$, SO$_4^{2-}$) (s, aq)	SFS-EN ISO 10304 Ion chromatography (Dionex ICS-1600 and IonPac AS4A-SC 4 x 250 mm or IonPac AS22 4 x 250 mm), solid samples extracted with 1:4 soil:water (w/v)	I–III
pH (s, aq)	SFS-ISO 10390 or SFS 3021, solid samples extracted 1:5 soil:0.01 M CaCl$_2$ (v/v)	I–III
E$_h$ (aq)	BlueLine 31 Rx Ag/AgCl electrode	III
EC (aq)	CEN/TS 15937, solid samples extracted 1:2.5 soil:water (v/v)	I
DOC (aq)	SFS-EN 1484, manual injections as in Stubbins & Dittmar (2012)	III–IV
DIC (aq)	Measured as CO$_2$ (g) from an acidified sample by a headspace technique (Trimmer et al., 2009)	III
Total Fe (aq)	Inductively coupled plasma spectroscopy by ALS Finland	III
CH$_4$, CO$_2$, N$_2$ (g)	Gas chromatography (Shimadzu GC-2014 with Agilent 80/100 Porapak N)	I–II
CH$_4$ (g)	Gas chromatography (PerkinElmer Clarus 500 with Supelco MOL Sieve 5A PLOT 30 m x 0.53 mm)	III
MPN of MOB and SRM (s)	Series of ten-fold dilutions of microbial culture medium MOB: Alexander, 1965 SRM: Stroes-Gascoyne et al., 2010; Bengtsson & Pedersen, 2017	II–III
ATP (s, aq)	Velten et al., 2007	III

s, solid sample; aq, aqueous sample, g, gaseous sample; TS, total solids; OM, organic matter; TOC, total organic carbon; WHC, water holding capacity; E$_h$, redox potential vs. standard hydrogen electrode; EC, electrical conductivity; DOC, dissolved organic carbon; DIC, dissolved inorganic carbon; MPN, most-probable number; MOB, methane-oxidizing bacteria; SRM, sulfate-reducing microorganisms; ATP, adenosine triphosphate.

Multiple linear regression (MLR) with forward stepwise selection of variables was used as a statistical method to identify the characteristics of the landfill cover soils on predicting the measured MOB activities. MOR was used as the dependent variable and nitrate and organic matter contents, pH of the material and basal respiration rate were studied as independent variables (linearity of the independent variables $0.3 < r < |0.7|$) (paper I). Student's 2-tailed t-test was used to assess the significance of the change in the MOB activities induced by different nutrient additions in the batch assay at confidence level of 95%. The critical t-value at two degrees of freedom was 4.303 (paper I).

In the continuous column experiment (Section 4.2.4), the methane elimination capacity (EC; g CH_4 m^{-2} d^{-1}) was calculated as a difference of the methane concentrations measured from the bottom inlet and top outlet of the column relative to the total surface area of the soil column (0.0158 m^2) (paper II). The methane removal efficiency (RE; %) was calculated as the amount of methane eliminated relative to the amount of methane inserted in the column (paper II).

The bulk density (ρ) of landfill soil in the columns and bentonite in the cells was calculated as wet mass of the material relative to volume (papers II–IV). The dry density of the bentonites (ρ_d) after the cell experiment were calculated based on the measured dry mass (m_s) and moisture contents (m_w) of the sample and literature values for the density of liquid (ρ_w) and bentonite particles (ρ_s) (Eq. 1; paper IV; Pusch, 2002):

$$\rho_d = \frac{m_s}{\frac{m_w}{\rho_w} \cdot \frac{m_s}{\rho_s}}$$

(1)

The total porosity (φ) of landfill soil in the columns was calculated based on the theoretical bulk density (ρ), measured weight fraction of wet solids (w_s) and density of landfill soil particles (Eq. 2; paper II; Mancebo & Hettiaratchi, 2015):

$$\varphi = 1 - \frac{\rho \cdot w_s}{\rho_s}$$

(2)

5   RESULTS AND DISCUSSION

The activity of MOB in selected landfill soil materials was studied in a set of batch assays and continuous column experiment and the activity of SRM and other co-existing microorganisms in the presence of bentonite materials in semi-continuous cell experiments and batch and leaching assays. The methane oxidation activities determined from a variety of landfill cover materials and their response to different improvement methods (fertilization, decompaction, amelioration with compost) are discussed in Section 5.1. The findings from the cell experiment used for studying both the role of bentonite organic matter as a substrate for the SRM and the effect of the microbial activity and other simulated repository conditions (compaction of bentonite to high dry density) on the quantity and release rate of bentonite sOM are presented in Section 5.2. Lastly, the factors identified to control the activity of MOB and SRM in the studied soil and bentonite materials are summarized in Section 5.3.

5.1   Methane oxidation activity in landfill cover structure soils

5.1.1   Factors governing MOB activity in different soils (paper I)

In total 12 soil samples collected from different locations and types of cover structures of two landfills were characterized chemically and tested for MORs by batch assays to identify soil factors governing the activity of MOB by a statistical MLR analysis.

It was found that all the screened soil samples were capable of oxidizing methane, but the level of activity differed notably between the samples collected from the cover structures of Ämmässuo and Tarastenjärvi landfills (Table 6). The MORs of compost-based biocovers of Ämmässuo landfill were approximately 10x higher (on average 4.16 µmol g^{-1} h^{-1}) than the MORs of the sand- and mineral soil-based cover structures of Tarastenjärvi landfill (on average 0.41 µmol g^{-1} h^{-1}, excluding sample Tj4; Table 6). The MORs observed for both the mineral- and compost-based soils were at the same range or slightly higher than the MORs reported by other studies

for composted garden and kitchen wastes considered as materials for biocovers (age 6–8 months, 7 µmol g^{-1} h^{-1}; Mor et al., 2006; Scheutz et al., 2014), compost-based landfill biocover material (age 5 years, 3 µmol g^{-1} h^{-1}; Einola et al., 2007) and different sandy and loamy landfill cover and other soil materials (0.01–1.0 µmol g^{-1} h^{-1}; Whalen et al., 1990; Kightley et al., 1995; Börjesson et al., 1998; Spokas & Bogner, 2011). The results indicate that the ageing compost can sustain MORs comparable to those of fresh compost for several years after installation (paper I).

The differences in the activities of MOB between the compost-based materials (from Ämmässuo) and mineral soils and sand (from Tarastenjärvi) could be explained by the characteristics of the soil materials for most of the samples (excluding Tj4, see below). In the compost-based materials, the organic matter content (5–28 % dry weight), moisture content (22–52 % wet weight), nitrate content (167–358 mg kg^{-1}) and sulfate content (125–8500 mg kg^{-1}) and basal respiration rates (0.05–2.01 µmol CO_2 g^{-1} h^{-1}) were on average higher than in the mineral soil-based materials (organic matter 3–5% dry weight, moisture 6–23 % wet weight, nitrate 14–52 mg kg^{-1}, sulfate <80 mg kg^{-1} except 1230 mg SO_4^{2-} kg^{-1} in the sand and basal respiration rate 0.04–0.50 µmol CO_2 g^{-1} h^{-1}; Table 6). The phosphate content or electrical conductivity of the soil materials did not deviate between the compost-based and mineral soils and the contents varied from <2 to 52 mg PO_4^{3-} kg^{-1} and from 52 to 2787 µS cm^{-1} (Table 6). The porosity and nutrient, organic matter and moisture contents are typically higher in compost-based materials than in mineral soils and these characteristics enable a higher gas exchange and availability of nutrients and, consequently, higher activity of MOB in the compost-based materials (Huber-Humer et al., 2009, 2011).

One exception to the discussion above was the mineral soil sample collected from Tarastenjärvi (Tj4), which had the highest MOR of all the samples (7.21 µmol g^{-1} h^{-1}), but lower nutrient, organic matter and moisture contents than many of the compost-based materials (Table 6). This suggests that the activity of MOB in the soils is affected also by some other factors, which were not included in the parameters studied here (paper I).

Table 6. Microbiological and chemical characteristics of landfill cover soils from Ämmässuo (As) and Tarastenjärvi (Tj) landfills (mean, $n = 2$–4) (adapted from paper I).

Sampling location[a]	Distributed[b] Year	Soil type[c]	MOR By MO	Prod$_{CO2}$ By MO	Prod$_{CO2}$ Via BR	pH	EC µS cm⁻¹	MC[d] % ww	OM % dw	NO$_3^-$ mg kg⁻¹	PO$_4^{3-}$ mg kg⁻¹	SO$_4^{2-}$ mg kg⁻¹
As1 10-20	2007	Compost	5.68	2.85	0.41	7.4	559	30	13	358	BD	125
As1 40-50	2007	Compost	5.17	2.51	0.34	6.9	496	22	5	232	BD	251
As2 10-20	2007	Compost	4.44	3.99	2.01	7.4	2787	38	22	18	52	7690
As2 40-50	2007	Compost	2.13	1.50	0.40	7.4	2020	31	14	14	BD	8519
As3 10-20	2010	Loamy compost	5.98	3.49	0.91	5.0	923	52	28	167	5.1	1393
As3 40-50	2010	Sandy compost	1.58	0.61	0.05	7.7	552	15	3	184	BD	449
Tj1	2007	Soil	0.20	0.16	0.09	7.3	291	16	6	23	BD	78
Tj2	2007	Soil	0.27	0.24	0.13	7.0	192	9	9	24	12	42
Tj3	2007	Sand	0.08	0.07	0.04	8.1	1176	6	3	52	BD	1234
Tj4	in 1990s	Soil	7.21	4.02	0.50	5.8	160	23	12	22	15	14
Tj5	in 1990s	Loamy soil	0.59	0.46	0.19	4.8	52	11	5	14	BD	14
Tj6	2012	Compost	0.91	0.97	0.63	7.0	1061	23	11	15	6.8	802

MOR, methane oxidation rate; Prod$_{CO2}$, CO$_2$ production rate; EC, electrical conductivity; MC, moisture content; OM, organic matter; MO, methane oxidation; BR, basal respiration; ww, wet weight; dw, dry weight; BD, below limit of detection (<2 mg kg⁻¹ dw).

[a]Samples from As were collected from depths 10-20 cm and 40-50 cm and for Tj from 5-20 cm.

[b]Samples were collected from Ämmässuo in 2012 and from Tarastenjärvi in 2013.

[c]Soil type categorized as "loamy compost" consisted of compost (70 %), fine stone aggregates (20 %) and clay (10 %); "sandy compost" consisted of compost (60 %) and fine stone aggregates (40 %); "loamy soil" consisted of soil (70 %) and clay (30 %).

[d]The moisture content of samples Tj1-3 and Tj5 was increased from the ambient to 33 % wet weight for determination of MOR.

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To explore the relationship of the MOB activities and the characteristics of the soil materials statistically, an MLR analysis was conducted, and the results showed that the basal respiration and the nitrate content were the significant factors predicting the activity of MOB (at 70%; Table 7). The relationship between the dependent and independent factors was positive i.e. the higher the basal respiration activity and nitrate content of the material, the higher the MOR (Table 7; paper I). The relationship of high soil nitrate content and activity of MOB has been highlighted also in a detailed study examining the effect of multiple characteristics of different compost and soil-based materials on the methane oxidation efficiency (Huber-Humer et al., 2011). The effect of high basal respiration on activity of MOB instead has been often reported to be inhibitive due to competition of heterotrophic bacteria and MOB over O_2 and, thus, the material of the biocovers is recommended to be mature enough to control the basal respiration (Mor et al., 2006; Huber-Humer et al., 2009, 2011). However, the results of the MLR analysis showed that the MOB could actually benefit from the concurrent modest activity of soil heterotrophic activity (Table 7; paper I). The co-existence of heterotrophic bacteria has been reported to benefit the activity of MOB also by others (e.g., Ho et al., 2014), but in this study, the relationship was shown to be statistically significant. The reason for the observed positive relationship can only be speculated, and it does not reveal the causality, but one explanation could be that the heterotrophic bacteria release beneficial compounds (e.g. nutrients) from soil organic matter for MOB to use (Ehrlich, 1996; Prescott et al., 2005).

Table 7. MLR of biocover soil characteristics and methane oxidation rates (MORs; paper I)[a]

	Standar-dised R	Unstan-dardised R	SE	Tolerance	t	p
(a) All variables included. $N = 12$, multiple $R^2 = 0.810$, adjusted multiple $R^2 = 0.701$, $F_{4,7} = 7.462$, $p = 0.011$.						
Intercept		3.226	0.807		3.999	0.005
NO_3^- (mg kg^{-1})	−0.609	−19.486	5.776	0.834	−3.374	0.012
BR ($\mu mol\ CO_2\ g^{-1}\ h^{-1}$)	0.985	1.685	0.532	0.281	3.167	0.016
OM ($\%_{dw}$)	−0.248	−0.028	0.034	0.307	−0.832	0.433
pH	0.146	0.683	0.875	0.781	0.781	0.461
(b) The best MLR model to predict variance in MOR data. $N = 12$, multiple $R^2 = 0.777$, adjusted multiple $R^2 = 0.728$, $F_{2,9} = 15.717$, $p < 0.001$.						
Intercept		2.914	0.303		9.609	< 0.001
NO_3^- (mg kg^{-1})	−0.547	−17.499	5.185	0.943	−3.375	0.008
BR ($\mu mol\ CO_2\ g^{-1}\ h^{-1}$)	0.835	1.429	0.277	0.943	5.154	0.001

MLR, multiple linear regression; SE, standard error; BR, basal respiration; OM, organic matter.
[a]Standardized and unstandardized correlation coefficients R with standard error. Tolerance as a measure of multiple correlations between variables. Data were square root transformed (MOR), $\log_{10}$ transformed (BR), inversed (NO_3^-) or inversed before $\log_{10}$ transformation (pH) to meet the assumptions of the statistical analysis.

5.1.2 Response of MOB activity to nutrient additions and other improvement methods (papers I and II)

The response of MOB to specific nutrient additions and different soil improvement methods (fertilization, decompaction and amelioration with compost) was studied to develop a method to enhance the activity of MOB in different landfill soil cover materials (compost-based and mineral). The efficiency of nutrient addition for enhancing the activity of MOB in particulate at low temperatures (4 °C and 12 °C) was studied in a batch assay (paper I), while all the three improvement methods were studied in a column experiment with continuous flux of artificial landfill gas and passive aeration (paper II). The compost sample from Ämmässuo (As2 40-50) and the soil sample from Tarastenjärvi landfills (Tj4; Table 6) were used in the batch assay and continuous column experiment, respectively, as justified earlier (Sections 4.2.3, 4.2.4).

In the batch assays, the activity of MOB in the Ämmässuo compost sample (As2 40-50) decreased with decreasing temperature from the initial 2.13 $\mu mol\ g^{-1}\ h^{-1}$ (determined at 21 °C; Table 6) to 0.499 $\mu mol\ g^{-1}\ h^{-1}$ at 12 °C and 0.090 $\mu mol\ g^{-1}\ h^{-1}$ at 4 °C (paper I). The decrease of the MOB activity with decreasing temperature was expected to be the case in the temperatures below the optimum temperature range of the MOB (20–35 °C; Einola et al., 2007, 2008; Spokas & Bogner, 2011). Some of the observed differences in the responses to the nutrient additions between the

samples were not statistically significant (Fig. 11), which could be resulting from the low number of parallel samples ($n = 2$). However, the greatest increment in activity of MOB, approximately by 16% versus the untreated control sample (0.090 µmol CH_4 g^{-1} h^{-1}), was achieved with the trace element and phosphorus addition at 4 °C (0.100 µmol CH_4 g^{-1} h^{-1}). At 12 °C, the trace element addition did not induce an increase in the activity of MOB compared to the activity in the control sample (Fig. 11b) and the reason could be that the psychrotolerant MOB (type I) expected to be active at 4 °C benefitted more from the addition of trace elements than the mixed community of MOB (types I and II) assumed to be active at 12 °C (Nikiema et al., 2013; Islam et al., 2020). To our knowledge, the role of trace elements and copper in particular in stimulating the activity of psychrotolerant MOB in landfill soil at low temperatures has not been brought into question before by other studies.
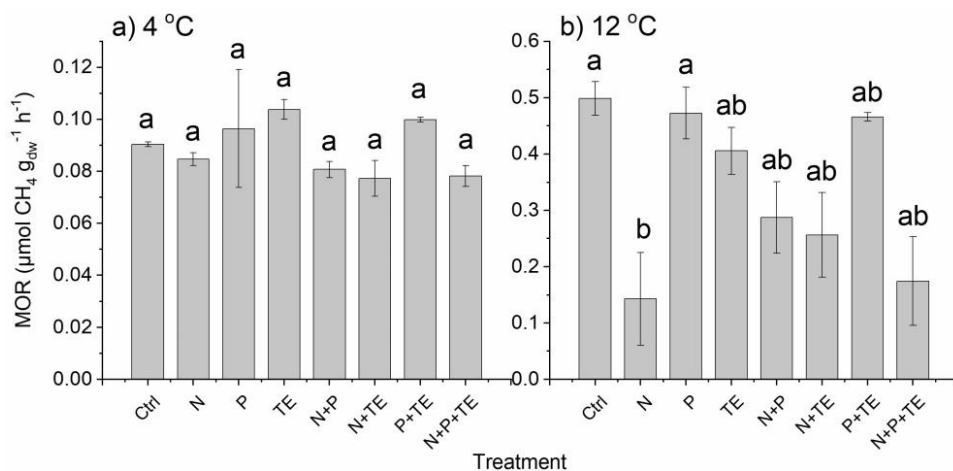
Figure 11. The effect of different nutrient additions on methane oxidation rates (MORs) of landfill cover samples relative to MOR in the control sample (Ctrl, no fertilizer; N, nitrogen; P, phosphorus; TE, trace elements) at a) 4 °C and b) 12 °C (mean ± standard error). Different small alphabets indicate statistically significant differences between mean values (Student's t-test; adapted from paper I)

Surprisingly, the fertilization with nitrate on its own or as a part of the fertilizer mixture affected the activities of MOB decreasingly at both temperatures (0.077–0.085 µmol CH_4 g^{-1} h^{-1} at 4 °C, 0.143–0.287 µmol CH_4 g^{-1} h^{-1} at 12 °C; paper I) relative to the control samples (Fig. 11; 0.090 µmol CH_4 g^{-1} h^{-1} at 4 °C, 0.499 µmol CH_4 g^{-1} h^{-1} at 12 °C) and the expected response was the opposite based on the results of the previous MLR analysis of the factors explaining the activity of MOB in the soil samples (Table 7). The hypothesized reason for this outcome was an unintentionally induced inhibition of the MOB by high ionic concentration of the

samples resulting from addition of fertilizers containing nitrate (approximately 10000 mg kg^{-1}, $\geq$2 mS cm^{-1}; paper I) as the inhibiting value for the salinity has been reported to be 4 mS cm^{-1} (Huber-Humer et al., 2009). Another possible reason is that the added nitrate itself decreased the activity of MOB in the soil samples here, similarly to some studies, which have reported a decrease in the MORs in the landfill soils after nitrate addition (by 64% after addition of 8850 mg NO_3^- kg^{-1} as NH_4NO_3: Kightley et al., 1995). Fertilization with phosphate was not shown to have an effect on the activity of MOB at 4 °C and 12 °C even though the concentration was increased from <2 mg kg^{-1} (Table 6) up to 1040 mg kg^{-1} to meet generously the theoretical phosphate requirement of the MOB ($\geq$55 mg kg^{-1}; paper I; Fig. 11).

In the column experiment (paper II), the improvement methods (fertilization, decompaction and amelioration with compost) were applied to the treatment column containing the mineral soil of Tarastenjärvi (Tj4; Table 6) at four occasions during the experiment. The control column was treated simultaneously, but instead of fertilization and compost amendment, the soil was irrigated with pure water and decompacted. The response of the activity of MOB to the improvement methods in the treatment column and to the parallel methods in the control column was observed during the experiment based on the methane EC. Before any improvement methods were applied, the methane EC of both columns was on average 55 g m^{-2} d^{-1} (RE of 55% at load of 100 g CH_4 m^{-2} d^{-1} and 24% at load of 210 g CH_4 m^{-2} d^{-1}; Fig. 12), which was slightly lower than reported earlier for similar setups containing sandy loamy landfill soil (Table 1; REs of 37–43% and 95–99% at inlet loads of 160 and 165 g m^{-2} d^{-1}, respectively; De Visscher et al., 1999; Huber-Humer et al., 2011). The methane load was increased from 110 to 210 g CH_4 m^{-2} d^{-1} on operation day 25 (Fig. 12) to exceed the methane EC of the landfill soil for testing the soil improvement methods (paper II).
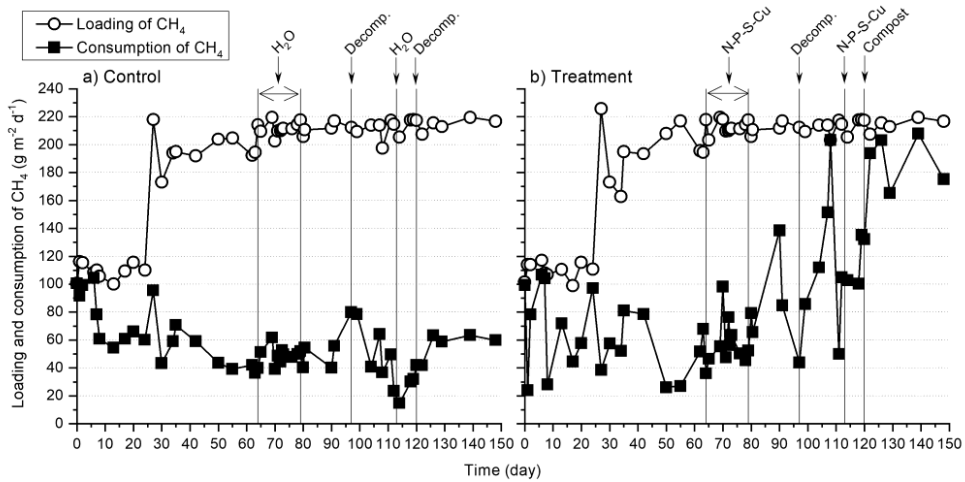
Figure 12. Methane loading and consumption rates and the applied improvement methods during the column experiment (Decomp., decompaction; adapted from paper II)

With the improvement methods, the greatest increase in the methane EC of the soil in the treatment column was achieved by ameliorating the soil with compost (22 w-%) at day 120; the methane EC increased to 189 g m^{-2} d^{-1} on average (RE 88%; Fig. 12b). This finding has novel practical significance as it provides a feasible method for controlling methane emissions for example from the existing cover structures of old landfills. The amount of compost addition required for increment of methane EC was notably lower than in other studies (Table 8). The other improvement methods applied to the soil in the treatment column, the first fertilization (days 64–79), decompaction (day 97) and the second fertilization (days 113–114), induced a subtler increase in the methane EC (to 138, 203 and 134 g m^{-2} d^{-1} corresponding to REs of 65, 100 and 61%, respectively), but the effect was short-term and the methane EC decreased at the level preceding the treatment (Fig. 12b). The methane EC in the control column did not respond notably to any of the applied improvement methods and the rate fluctuated between 35 and 55 g m^{-2} d^{-1} throughout the experiment (RE 16–27%; Fig. 12a; paper II).

Table 8. Comparison of effect of different improvement methods on methane elimination capacities of landfill cover soils and cover materials in continuous laboratory column experiments with passive aeration (adapted from paper II)

Material	Soil type	Improvement	MC (% ww)	WHC (% dw)	OM (% dw)	Dry density (kg L⁻¹)	Inlet load (g m⁻² d⁻¹)	EC (g m⁻² d⁻¹)	RE (%)	Ref.
Landfill soil	Silty gravelly sand (39.8% gravel, 49.9% sand, 10.3% silt)	637 mg NO$_3^-$ kg^{-1} (22[a]) 425 mg PO$_4^{3-}$ kg^{-1} (15[a]) 0.53 mg Cu^{2+} kg^{-1} (0.11[a]) 1180 mg SO$_4^{2-}$ kg^{-1} (14[a])	34	79	9.9	0.89	210	87 (45-203)[b]	41 (21-100)[b]	This study
Soil	Loamy sand	44, 111 or 221 mg NO$_3^-$ kg^{-1} (3[a]) +80 mg P kg^{-1} (21[a]) +150 mg K kg^{-1} (40[a])	13	n.r.	0.4	1.61	526	428 (44 mg NO3-) 436 (111 mg NO3) 432 (221 mg NO3)	81 83 82	[1]
Landfill soil	Coarse sand (70% coarse sand, 18% fine sand, 16% silt/clay)	Control 2000 mg N kg^{-1} as NH$_4$NO$_3$ (n.r.[a]) 100 mg P kg^{-1} as K$_2$HPO$_4$ (n.r.[a]) 100 mg P+100 mg N kg^{-1} as SeSl	11±7	55	~0.8	n.r.	271	92 (Control) 33 (NH4NO3) 92 (K2HPO4) 116 (SS)	34 12 34 43	[2]
Landfill soil	Silty gravelly sand (39.8% gravel, 49.9% sand, 10.3% silt)	Decompaction	35	79	10.5	0.83	210	47[c]	22[c]	This study
Leaf compost	Particle size <5 mm	Decompaction	55	n.r.	46	0.64[d]	520	100→500	19→95	[3]
Landfill soil	Silty gravelly sand (39.8% gravel, 49.9% sand, 10.3% silt)	Compost 22 w-%	41	110	17.6	0.64	210	189[e]	88[e]	This study
Landfill soil	Silty clayey soil (30% sand, 30% silt, 40% clay) + compost (<10 mm)	Compost 25 w-% Compost 50 w-%	19 22	n.r.	n.r.	1.39 1.21	680	137 175	20 26	[4][f]
Garden soil	Soil type n.r. + compost (<20 mm or <15 mm)	Compost 60 w-%	30-47	60-79	14-16	1.14-1.20[d]	165	149-165	90-100	[5]

MC, moisture content; dw, dry weight; ww, wet weight; WHC, water holding capacity; OM, organic matter; EC, elimination capacity; RE, removal efficiency; n.r., not reported; SeSl, sewage sludge; w-%, percentage by weight. References: [1] Park et al., 2002; [2] Kightley et al., 1995; [3] Wilshusen et al., 2004; [4] Rose et al., 2012; [5] Huber-Humer et al., 2011.

[a]Initial soil nutrient concentration. [b]Treatment column, days 65–120; average (min–max). [c]Control column, days 98–148. [d]The nature of the density (wet or dry) was not specified by the authors. [e]Treatment column, days 120–148. [f]Active aeration from the bottom of the column.

The increasing effect of the compost amendment on the methane EC of the treatment column soil was explained by the changes in the characteristics of the soil-compost mixture relative to the characteristics of the mineral soil. In the soil-compost mixture, WHC had increased from 79 to 110%, moisture content from 34 to 41% wet weight, organic matter content from 10 to 17%, total porosity from 0.64 to 0.70 L L^{-1}, activity of MOB determined in a batch assay from 30 to 59 µg CH$_4$ g^{-1} h^{-1} and most probable number (MPN) of the MOB from $13 \cdot 10^6$ to $705 \cdot 10^6$ cells g^{-1}, while dry density decreased from 0.86 to 0.64 kg L^{-1} (Table 9). Also, the nutrient content of the soil increased following from compost amelioration (Table 9). One parameter not included in the present study, water potential, could have been used for studying more in detail the combined effect of the different parameters, that is water availability (matric potential) and concentration of salts in the porewater (osmotic potential), on the activity of MOB in the soil and soil-compost mixture (Chowdhury et al., 2011).

The increase in the WHC following from increased concentration of organic matter and porosity of the soil (Bohn et al., 2011; Olorunfemi et al., 2016) facilitated the diffusion of gases, which was detected as increased concentration of N$_2$ at every depth of the soil bed in the treatment column after amelioration with compost (from 14–39 to 35–65 v-%; Fig. 13). Gaseous nitrogen was considered as a representative gas for estimating gas diffusion properties of the soil as it is not affected by the methanotrophic metabolism (Humer & Lechner, 1999). The increased diffusion of gases and multiplied number of MOB in the treatment column soil-compost mixture led to a twice higher MOR than in the unamended soil of the control column (Table 9), which undeniably increased the methane EC of the soil bed in the treatment column (paper II). The methanotrophs could have also benefitted from the increase of nutrients introduced by the compost as the organic fertilization has been shown to benefit the methanotrophs more than inorganic fertilization in agricultural soil (Seghers et al., 2003).

Table 9. Chemical and biological characteristics of the landfill cover soil, compost, and soil-compost mixture in the laboratory columns at different times of the experiment[a] (mean ± standard deviation, n = 1–3) (adapted from paper II)

Parameter	Cover soil	Compost	Control			Treatment		
Day	0	0	97	120	148	97	120	148
Dry density of the soil (kg L⁻¹)	n.a.	n.a.	0.87	0.86	0.83	0.87	0.86	0.64
Total porosity (φ)	n.a.	n.a.	0.64	0.64	0.65	0.64	0.64	0.70
pH	5.84 ±0.054	6.87 ±0.007	6.23 ±0.041	6.27 ±0.106	6.31 ±0.048	6.44 ±0.083	6.68 ±0.035	6.91 ±0.050
WHC (% dw)	79	218 ±1.3	79	79	79	79	79	110
MC (% ww)	33.6 ±2.17	56.7 ±2.29	36.8 ±1.35	33.8 ±0.30	34.3 ±1.74	31.8 ±0.69	35.7 ±2.11	40.8 ±2.29
MC (% WHC)	42.6	26.0	46.6	42.8	43.4	40.2	45.2	37.2
OM (% dw)	10.4 ±0.82	41.9 ±5.30	11.4 ±0.48	10.1 ±0.26	10.1 ±0.70	9.1 ±0.26	10.7 ±0.86	17.0 ±5.30
NO₃⁻ (mg kg⁻¹)	22.3 ±1.05	6279 ±93.9	51.5 ±5.83	42.6 ±0.53	22.1 ±4.77	62.6 ±18.32	69.5 ±19.29	950 ±554.90
PO₄³⁻ (mg kg⁻¹)	14.8 ±0.77	BD	6.7 ±1.76	6.9 ±1.48	6.0 ±1.15	31.4 ±9.60	61.2 ±16.21	71.3 ±5.61
SO₄²⁻ (mg kg⁻¹)	14.3 ±0.20	2947 ±227.5	14.8 ±3.69	14.9 ±1.19	18.1 ±7.75	482 ±67.3	1092 ±32.1	1585 ±123.5
Cu²⁺ (mg kg⁻¹)	0.114 ±0.057	0.231 ±0.006	0.028 ±0.019	0.149 ±0.001	0.093 ±0.029	0.193 ±0.002	0.262 ±0.008	0.270 ±0.003
MPN (· 10⁶ cells g⁻¹)	5.9	29.6	3.1	n.m.	6.4	13.0	n.m.	705
MOR mean (µg CH₄ g⁻¹ h⁻¹)	25.7 ±2.97	135.9 ±20.65	29.0 ±4.46	n.m.	27.0 ±6.36	30.1 ±7.08	n.m.	58.5 ±33.75

n.a., not applicable; WHC, water holding capacity; MC, moisture content; OM, organic matter content; dw, dry weight; ww, wet weight; n.a., not applicable; n.m., not measured; MPN, most-probable number of methane-oxidizing bacteria; MOR, methane oxidation rate. The control column was subjected to irrigation and decompaction, and the treatment column to fertilisation and compost addition before sampling at days 97 and 148, respectively.

[a]Samples analyzed before the start of the experiment (day 0), after fertilization of treatment column or irrigation of control column (day 97), after decompaction (day 120) and after compost addition to treatment column or decompaction of control column (day 148).
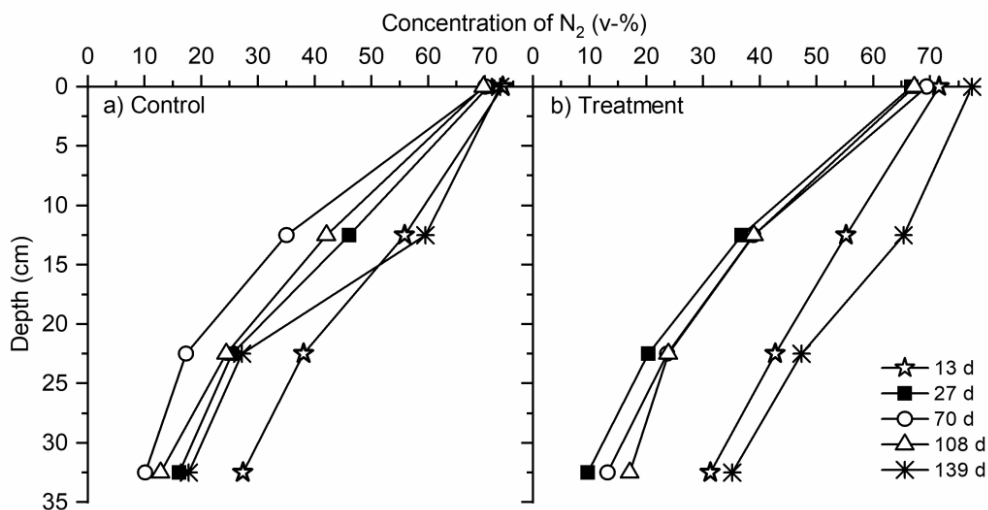
Figure 13. Concentration of N₂ over the depth of control (C) and treatment (T) columns measured before application of any methods (days 13 and 27), during irrigation (C) or fertilization (T) and before decompaction (C+T; day 70), before decompaction (C) or amelioration with compost (T) (day 108), and at the end of the experiment after decompaction (C) or compost addition (T) (day 139) (adapted from paper II)

The effect of fertilization and decompaction on the methane EC of the soil in the treatment column was low (Fig. 10b) contrary to expectation based on the MLR analysis (Table 7) and the fine particle size of the mineral soil (paper II), which suggested that increasing the porosity by decompaction and nitrate content by fertilization would increase the methane EC. The fertilization occasions indeed increased the number of MOB (from $6 \cdot 10^6$ to $13 \cdot 10^6$ cells g⁻¹; Table 9) and methane EC shortly (Fig. 12b), but the analysis of the nutrient content of the soil in the treatment column (at day 120, before compost amelioration) revealed that the mineral soil was so nutrient-deprived that the added fertilization had been used up very quickly (Table 9) and, thus, the addition did not support a long-lasting increase in the methane EC (paper II). Other studies conducted with mineral landfill cover soils and other soils (Kightley et al., 1995; Park et al., 2002), have similarly reported a low response of MOB activity to inorganic fertilization (Table 8). As for decompaction, the likely reason why the method was inefficient to accelerate methane EC was that the gas diffusion properties of the soil columns did not deteriorate during the experiment, as could be concluded from the unchanged N₂ concentration profiles of the soil columns between days 27 and 108 (Fig. 13). In other studies (Wilshusen et al., 2004), a compost column operated a high methane load was found to become clogged due to bacterial biomass formation and, thus,

decompaction of the soil restored the methane EC (Table 8). Here, the slight increase of the methane EC of the treatment column after decompaction (Fig. 12b) was likely resulting from even distribution of the locally added nutrients and colonized methanotrophic cells detected as coloured zones in the mid-height of the soil beds (Humer & Lechner, 1999) instead of improvement of diffusion of gases (paper II).

5.2 Interaction of microorganisms and bentonite organic matter in the simulated repository conditions

5.2.1 Growth of SRM and other microorganisms on bentonite organic matter (papers III and IV)

The growth of microorganisms on the bentonite organic matter was studied with the cell experiment and the post-experimental batch assays. In the cell experiment, the repository conditions were simulated by compacting the studied bentonites to target density of 1400 kg m^{-3} (resulting densities 1314–1368 kg m^{-3}) and by connecting them to a highly permeable sand layer, which was either inoculated or uninoculated with external microorganisms. Unsterilized bentonites were known to contain indigenous microorganisms, whose activation was likely during the experiment (paper III).

The concentrations of DOC, DIC, sulfate and iron were measured from the sand layer solutions of the six cells during the experiment, and the differences in the evolution of the concentrations between the inoculated and uninoculated cells could be used to conclude the activity of different microorganisms in the cells. The only organic substrate provided for the SRM and other microorganisms in the cells was the organic matter released by the bentonites and the DOC concentration in the sand layer solution remained at the range of 2–23 mg L^{-1} on average throughout the experiment (Fig. 14a–c). The organic matter released by the bentonites in the inoculated cells could have been also contributed by microbiologically produced compounds transmitted to the cells by the inoculated sand (Muyzer & Stams, 2008; Decho & Gutierrez, 2017), but this contribution was believed to be negligible as the concentration of DOC was initially lower in the inoculated cells of the Wyoming and Indian bentonites than in the respective uninoculated cells (Fig. 14a, b; paper III). During the experiment, the average DOC concentration in the sand layers of the inoculated Wyoming and Indian cells decreased to lower level than in the

uninoculated cells (Fig. 14a, b). This difference suggested that the organic matter was consumed microbiologically in the inoculated cells, yet the microbiological consumption in the uninoculated cells could not be completely excluded (paper III). In the cells of the Bulgarian bentonite, the DOC concentrations showed the opposite trend (higher in inoculated than uninoculated) for an unknown reason.
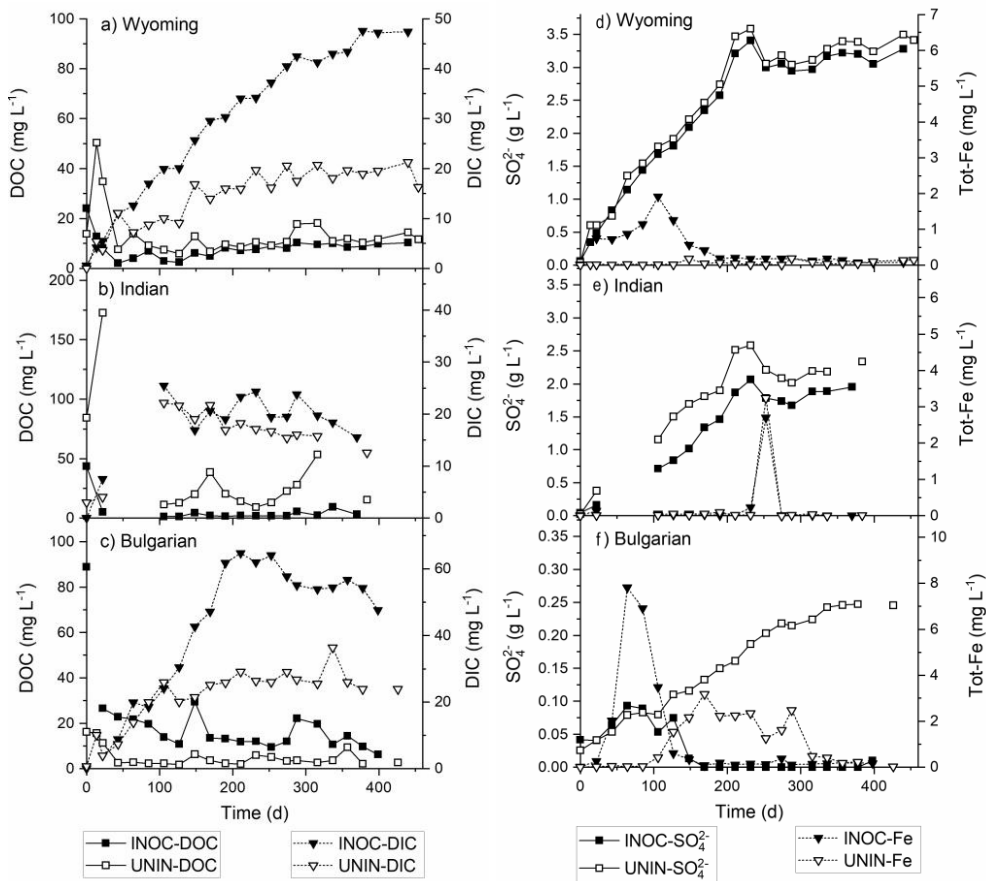
Figure 14. Dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), sulfate and total iron in the sand layer solutions of experimental cells inoculated (INOC) or uninoculated (UNIN) with microorganisms containing different bentonites (a–c and d–f). Note the different scales on the y-axes (adapted from paper III)

Concentration of DIC increased in the sand layer solution of all the cells during the cell experiment because of calcite dissolution from the bentonites (Melamed & Pitkänen, 1996) and because of microbiological respiration, which was the likely reason for the concentration of DIC being higher in the inoculated cells than in the uninoculated cells (Fig. 14a–c). Gypsum dissolving from the bentonites (Melamed

& Pitkänen, 1996) increased the concentration of sulfate up to 3.2, 2.1 and 0.125 g L^{-1} in the sand layer solutions of the uninoculated cells of Wyoming, Indian and Bulgarian bentonites, but the concentrations observed in the inoculated cells were approximately by 250–680 mg L^{-1} lower than in the uninoculated cells because of the activity of SRM (Fig. 14d–f). The concentration of total iron increased in the sand layer solution of all the inoculated cells (up to 1.9, 0.13, and 7.8 mg L^{-1} in Wyoming, Indian, and Bulgarian bentonites) and also in the uninoculated cell of the Bulgarian bentonite (2.1 mg L^{-1}) presumably due to activity of such microorganisms (e.g., iron-reducing bacteria, IRB), which can promote dissolution of iron from the bentonites (Fig. 14d–f; Colombo et al., 2013). The observed decrease of iron concentration (Fig. 14d–f) instead was resulting from the concomitant activity of SRM and the produced sulfide, which precipitated with iron as iron sulfides (e.g. FeS and Fe_3S_4) identified from the sand layers of the inoculated cells of the Wyoming and Bulgarian bentonites (paper III). As for methane, it was detected from all the six cells at concentrations ranging from <10 μg L^{-1} to <650 μg L^{-1} indicating activity of methanogens in all cells (paper III). In the inoculated cells, the SRM, methanogens and IRB could have originated from the microbial community added to the cells in the beginning of the experiment, but in the uninoculated cells their origin was likely the bentonites, which have reported to contain several indigenous microorganisms including SRM, methanogens and IRB (Masurat et al., 2010; Svensson et al., 2011; Stroes-Gascoyne et al., 2014).

The activities of overall microbial community and the SRM in the sand layers of the cells was confirmed post-experiment by measuring the ATP concentration and determining the SRRs in batch assays. The overall microbial activities were higher in the inoculated cells and lower in the uninoculated cells after the experiments than before the experiment based on the ATP concentrations (Table 10; paper III). The overall microbial activity (8.1 nmol ATP L^{-1}) was the highest in the inoculated cell of the Bulgarian bentonite suggesting that the organic matter released by the Bulgarian bentonite (or other conditions resulting from the presence of that bentonite) supported higher microbial activity than organic matter of the other bentonites. However, the low SRRs in the sand of the inoculated cell of the Bulgarian bentonite (0.79 mg SO_4^{2-} L^{-1} d^{-1}) indicated that the overall microbial activity detected at the end of the experiment (based on ATP concentration) could be attributed to the activity of other microorganisms than SRM (Table 10; paper III). In the inoculated cells of the Wyoming and Indian bentonites, the overall microbial activities (2.0 and 2.3 nmol ATP L^{-1}) were lower but the SRRs (2.0 and 2.1 mg SO_4^{2-} L^{-1} d^{-1}) were higher than in the inoculated cell of the Bulgarian bentonite, which

indicated that in these cells the overall microbial activity was dominated by the SRM (Table 10; paper III). The overall microbial activity detected in the sand layer of the uninoculated cell of the Bulgarian bentonite (0.38 nmol ATP L^{-1}) could be partly attributed to the activity of indigenous SRM (SRR 0.94 mg SO_4^{2-} L^{-1} d^{-1}), while in the uninoculated cells of the other bentonites, no activity of indigenous SRM could be detected in the post-experiment batch assay based on the SRRs (Table 10). The uninoculated cell of the Indian bentonite was distinguishable from the other uninoculated cells by showing practically no overall microbial activity in the sand layer (<0.0002 nmol ATP L^{-1}; Table 10) suggesting that some of the active microorganisms (e.g. methanogens) were located inside the bentonite block instead of the sand layer (paper III).

Table 10. ATP concentrations and SRRs in the sand and solution of the experimental cells with different bentonites (mean ± SD, n = 2–3) (adapted from paper III)

Cell	Bentonite	ATP (nmol L^{-1})	SRR (mg SO_4^{2-} L^{-1} d^{-1})
AGW-based solution added in the cells in the beginning of the experiment			
UNIN	Initial all	<0.09	n.m.
INOC	Initial all	2.8 ± 0.08	n.m.
Cell	**Bentonite**	**ATP (nmol kg^{-1})**	**SRR (mg SO_4^{2-} g^{-1} d^{-1})**
Sand added in the cells in the beginning of the experiment			
UNIN	Initial all	0.20 ± 0.037	n.m.
INOC	Initial all	0.35 ± 0.034	n.m.
Sand collected from the cells at the end of the experiment			
UNIN	Wyoming	0.13 ± 0.075	0
	Indian	<0.0002	0
	Bulgarian	0.38 ± 0.098	0.94 ± 0.260
INOC	Wyoming	2.0 ± 0.13	2.0 ± 0.59
	Indian	2.3 ± 0.27	2.1 ± 0.40
	Bulgarian	8.1 ± 0.83	0.79 ± 0.257

ATP, adenosine triphosphate; SRR, sulfate reduction rate; SD, standard deviation; AGW, artificial groundwater; UNIN and INOC, the sand layers of the cells were uninoculated or inoculated with microorganisms; n.m., not measured.

The exact location of the active microorganisms inside the cells (sand layers or bentonite blocks) could not be verified based on the measured data, but the post-experiment determination of the dry density distribution and number of SRM (by the MPN technique) in the bentonite blocks revealed that the bentonite densities were not high enough at least in the top layers to inhibit the activity of microorganisms within the six bentonite blocks (paper IV). In all blocks, the dry density of the top four centimeters of the 16 cm high bentonite blocks were by 30–

200 kg m^{-3} lower than the dry densities on the bottom of the blocks resulting in density ranges of 1140–1330 kg m^{-3} at the surface (0–4 cm) and 1270–1380 kg m^{-3} at the bottom of the blocks (Fig. 15). To restrict the activity of SRM, the dry densities of the Wyoming and Indian bentonites should have been higher than 1350 and 1280 kg m^{-3}, respectively (Bengtsson & Pedersen, 2017; paper IV), while no such threshold density has been reported for the Bulgarian bentonite. However, the swelling pressure of the Bulgarian Ca-bentonite is known to be considerably higher than that of the Wyoming and Indian Na-bentonites (Karnland et al., 2006; Kumpulainen et al., 2016; Svensson et al., 2019), so the swelling pressure could have limited the activity of microorganisms at least in deeper layers of the Bulgarian bentonite (paper IV). Generally, the swelling capacity of a Ca-bentonite could be expected to be lower than that of a Na-bentonite due to the lower number of hydrated divalent cation layers, but Bulgarian bentonite is an exception for a yet unknown reason (Schanz & Tripathy, 2009; Svensson et al., 2019).

The assumption of swelling pressure restricting the growth of microorganisms in the Bulgarian bentonite blocks is supported by the decrease of the MPN of SRM in both blocks of the Bulgarian bentonite during the experiment (1200–2200 SRM g^{-1} of bentonite after the experiment vs. 3800 SRM g^{-1} in the original Bulgarian bentonite) because in the other bentonites, the number of SRM had increased during the experiment (Table 11; paper III). However, the reactive transport calculations predicting evolution of sulfate and DOC in the sand layers of the Bulgarian cells indicated that SRM had been active at least in the top depths of the bentonite blocks in both cells (Kiczka et al., 2021). For the Indian bentonite, the active microorganisms locating inside the bentonite blocks would explain the observed activity of SRM in the inoculated cell of the Indian bentonite. That cell never reached a low enough redox potential (>200 mV vs. standard hydrogen electrode) to enable activity of the SRM in the sand layer while the other inoculated cells did (up to -150 mV on average; Frindte et al., 2015), yet the results indicated that sulfate reduction had occurred in the inoculated cell of the Indian bentonite (paper III).
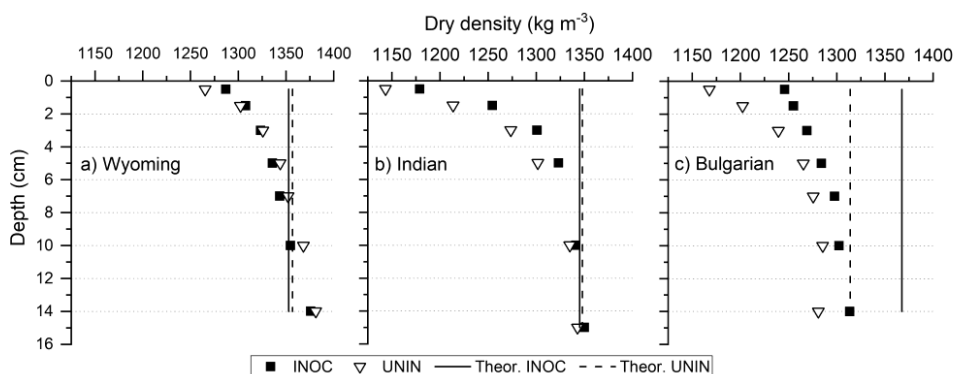
Figure 15. Vertical distribution of the dry densities in the bentonite blocks of the cells at the end of the experiment (INOC, inoculated; UNIN, uninoculated; Theor., theoretical density calculated from the volume and dry mass of the bentonite in the cell) (paper IV)

Table 11. MPN of SRM in the bentonite before and after the cell experiment (paper III)

Bentonite	Cell	MPN of SRM (g^{-1} bentonite) (lower–upper 95% confidence interval)	
Wyoming	Original	370	(100–1300)
	UNIN	210	(72–620)
	INOC	660	(160–2800)
Indian	Original	7500	(1900–30 000)
	UNIN	2900	(1100–7900)
	INOC	14 000	(4100–47 000)
Bulgarian	Original	3800	(100–14 000)
	UNIN	2200	(750–6500)
	INOC	1200	(360–3800)

MPN, most probable number; SRM, sulfate-reducing microorganisms; UNIN and INOC, sand layer of the cells were uninoculated or inoculated with microorganisms.

5.2.2 Effect of simulated repository conditions on bentonite soluble organic matter (papers III and IV)

To assess the changes in the bentonite sOM induced by the simulated repository conditions, the quantity and release rate of the bentonite sOM was determined for the original bentonites and for the bentonites after the cell experiment by dynamic leaching assays. The sOM contents of the original bentonites varied between the bentonites; 85, 16 and >163 mg kg^{-1} for Wyoming, Indian and Bulgarian bentonites, respectively (Fig. 16, Table 12). The results indicate that only a small part of organic

matter in bentonites was originally water-soluble (4.2, 2.4 and >11.6% of total organic carbon [TOC], respectively; Table 12) even when the interaction of the bentonite with water was maximized by high L/S ratio in the dynamic leaching (paper IV). The results agreed with the sOM content range reported for the Wyoming-type bentonite in the literature (approximately 16–90 mg kg^{-1} corresponding to 2–8% of TOC; Marshall et al., 2015; Usman & Simpson, 2020), while this was the first work reporting the sOM contents of the Indian and Bulgarian bentonites (paper IV).

The results of the post-experiment dynamic leaching assay showed that exposing the bentonites to simulated repository conditions during the cell experiment had increased the bentonite sOM contents and/or release rates compared to the original bentonites. The average sOM content of the Indian bentonite in both cells (22 and 61 mg kg^{-1} or 3.3 and 9.0% of TOC for inoculated an uninoculated cells) and Bulgarian bentonite in the inoculated cell (270 mg kg^{-1}, 19.3% of TOC) had increased during the experiment relative to the original bentonites (Table 12). For the Wyoming bentonite in both cells (52 and 68 mg kg^{-1} or 2.6 and 3.4% of TOC in inoculated and uninoculated cells) and for Bulgarian bentonite in the uninoculated cell (47 mg kg^{-1} or 3.3% of TOC), the sOM content was on average lower than in the original bentonites (Table 12) possibly because of microbial consumption of sOM or because the leaching was stopped before exhaustion of the sOM release was achieved for some of the samples (paper IV). The release rate of the bentonite sOM instead had increased in all bentonites after the cell experiment compared to the original bentonites (paper IV). This could be concluded based on the results from the dynamic leaching assays; ≥80% of the sOM became released from the bentonites exposed to simulated repository conditions within 1–4 leaching cycles, while the original bentonites required up to 19 leaching cycles to reach the same proportional release of sOM (Fig. 16; paper IV).

Table 12. Soluble organic matter (sOM) contents (mg kg⁻¹)[a] of the original bentonites and bentonites exposed to simulated repository conditions determined by dynamic leaching assays (mean ± standard deviation, n = 3–4) (paper IV)

Bentonite	Wyoming		Indian		Bulgarian	
Original (as received)	85 ±9.0		16 ± 6.4		163† ± 15.5	
Relative to TOC (%)[b]	4.2		2.4		11.6	
Cell ▲	UNIN	INOC	UNIN	INOC	UNIN	INOC
Depth from the sinter ▼						
0–1 cm	36 ±2.6	26 ±5.1	65 ±2.2	32 ±4.7	31† ±12.0	62 ±9.9
1–2 cm	60† ±12.9	24 ±0.3	64 ±7.6	29 ±4.9	46† ±9.4	303 ±21
2–4 cm	57† ±6.8	42 ±4.8	64 ±2.2	24 ±5.0	46† ±16.5	264 ±6.1
4–6 cm	65† ±6.9	76† ±8.0	58 ±2.9	22 ±4.4	47† ±3.1	274 ±1.4
6–8 cm	46 ±9.3	73† ±5.4	n.a.	n.a.	53† ±8.6	283 ±32
8–12 cm	47 ±4.7	84† ±11.3	n.a.	n.a.	46† ±13.8	307 ±12
9–11 cm	n.a.	n.a.	64 ±19.5	19 ±1.4	n.a.	n.a.
12–16 cm	54† ±11.6	78† ±8.0	n.a.	n.a.	48† ±8.9	279 ±8.6
14–16 cm	n.a.	n.a.	54 ±18.4	16 ±2.1	n.a.	n.a.
Weighted average all depths[c]	52	68	61	22	47	270
Relative to TOC (%)[b]	2.6	3.4	9.0	3.3	3.3	19.3

UNIN and INOC, cells in where the sand layers were uninoculated or inoculated with microorganisms; n.a., not applicable; TOC, total organic carbon.

[a]sOM contents normalized to dry mass of the sample.

[b]sOM contents given relative to the TOC content of the original bentonites (2023, 680 and 1400 mg kg⁻¹ for Wyoming, Indian and Bulgarian bentonites, respectively; Table 4).

[c]The mass factor of each depth layer has been taken into account in calculation of the average values for the entire bentonite blocks (paper IV).

†The exhaustion of sOM release from the sample was not reached during the assay, so the actual sOM quantity was likely to be higher than reported.

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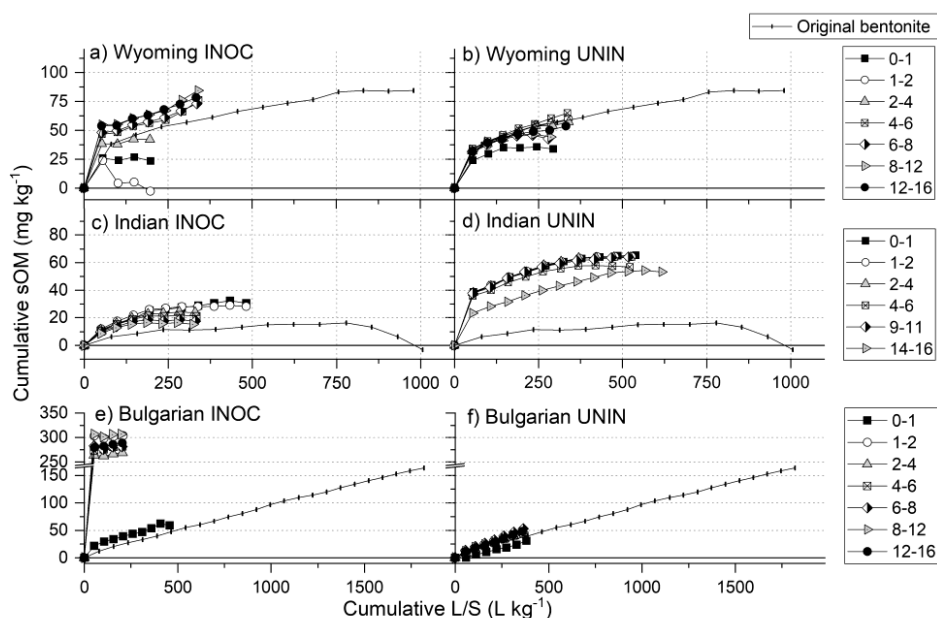
Figure 16. Cumulative amount of soluble organic matter (sOM) released by the bentonites before (Original as received) and after exposing the bentonites to the simulated repository conditions in cells uninoculated (UNIN) or inoculated (INOC) with microorganisms (bentonite sampled from different depths of the blocks e.g., 0–1 cm) as a function of cumulative liquid-to-solid (L/S) ratio (mean, n = 2–4). Note different scales on the axes (paper IV)

As indicated by the results of the dynamic leaching assays, the simulated repository conditions increased the sOM quantity (Indian and Bulgarian bentonites) and release rate (all bentonites) of the exposed bentonites compared to the original bentonites. Because also the uninoculated cells were shown to contain active microorganisms (indigenous to bentonites; Section 5.2.1), the effect of microbial activity could not be separated from the effect of abiotic factors i.e. bentonite compaction on bentonite sOM. It is hypothesized that in compaction, the bentonite microstructure changes, and the inter-aggregate porosity decreases Delage et al., 2006; Likos & Wayllace, 2010), which results in breakage of the smectite microaggregates and consequent release of organic matter (paper IV). However, the quantity of sOM in individual depth layers did not seem to correlate with the compaction degree i.e. the dry density of the layers (Table 12, Fig. 15), which suggests that there were other processes, such as microbial activity, that increased the sOM quantity in the exposed bentonites relative to the original bentonites (paper IV).

The distribution of the sOM quantities of the exposed bentonites relative to the corresponding sampling depths showed that the sOM quantity was lower in the

bentonite below the sinter than on the bottom of the blocks in the inoculated (by 142 mg kg^{-1}, 0–4 cm) and uninoculated (by 19 mg kg^{-1}, 0–1 cm) cells of Wyoming bentonite and in the inoculated cell of the Bulgarian bentonite (by 223 mg kg^{-1}, 0–1 cm; Table 12). The depletion of sOM from the surface of the Wyoming and Bulgarian bentonite blocks was an additional evidence of microbial activity within the cells: the concentration of DOC detected from the sand layer solutions of these cells during the experiment (2–23 mg L^{-1}; Fig. 14a, c) corresponded only to 1–10 mg sOM kg^{-1} relative to bentonite at depths of 0–4 cm (paper IV), so majority of the sOM missing from the top depths of the blocks of Wyoming and Bulgarian bentonites had been consumed by the microorganisms. For the cells with Indian bentonite, similar depletion of sOM from the surface layers of the blocks could not be detected.

The sOM contents and release rates of the bentonites from the inoculated cells being higher than those of the bentonites from the uninoculated cells (Fig. 16, Table 12) supported the assumption that microbial activity increased sOM quantity and release rate of the exposed bentonite. The exact mechanism of microbial interaction with bentonite could only be hypothesized, but the quantity of sOM could be increased by release of organic matter from the smectite surfaces after alteration of the bentonite structure via structural iron reduction by SRM, IRB and/or methanogens (Zhang et al., 2014; Cuadros, 2017; Broz, 2020), which were shown to be active in the cells (Section 5.2.1). Another possibility is that microorganisms had degraded the bentonite organic matter to smaller, more easily desorbed, and transported compounds (Arnarson & Keil, 2000; Condron et al., 2010; Durce et al., 2018) (paper IV). Transport of the organic matter from the bentonite blocks by diffusion during the cell experiment was determined by numerically by Kiczka et al. (2021).

5.3 Overview on controls of microbial activity in the engineered barriers

The two main studied microbial processes, methane oxidation and sulfate reduction, differ from each other in a way that the former is a desirable and often technically promoted process to control methane emissions from the MSW landfills, while the latter is an unwanted but unavoidable naturally occurring process in the SNF repository environment (Huber-Humer et al., 2008; Pedersen et al., 2008). However, as can be interpreted from the results of this thesis, the activity of both microbial

groups is dependent on the physicochemical and also biological properties of the engineered barrier materials and, thus, they can be either enhanced (methane oxidation) or restricted (sulfate reduction) by manipulating some of the material properties as discussed below (Table 13).

The results from the column and cell experiments show that the transport and supply of the essential substrates (methane and oxygen for the MOB, and organic matter and sulfate for the SRM) at the reach of the microorganisms affected greatly their activity, and that the transport of the compounds through the barrier materials was mostly governed by the density and, hence, the porosity of the barrier materials (papers II–IV). For the landfill cover soil, the significance of the soil physical properties on the transport of methane could be concluded based on the increased methane EC of the mineral soil after addition of compost (22 w-%), which resulted in a decreased bulk density and increased total porosity and WHC of the soil-compost mixtures enabling higher gas diffusion (paper II). As for the bentonites, the release of organic matter from the loosely arranged bentonites to the leachants at high L/S ratio (195–1819 L kg^{-1}) in the dynamic leaching assays was considerably faster than from the compacted bentonites to the external water body (e.g. groundwater) (paper IV). In compacted bentonites the transport is controlled by diffusion, which again is affected by surface charges of the smectites, density and anion accessible porosity of the bentonite and ionic strength of the pore water (Alt-Epping et al., 2015; Durce et al., 2018). Even though it was not shown experimentally, it is justified to assume that the microbial activity during the cell experiment was limited by the supply of organic substrates from the bentonites (paper III). As a conclusion, the activity of the microorganisms could be increased or decreased by controlling the supply of substrates via density and porosity of the barrier materials (Table 13).

Table 13. Summary of identified effective factors and suggested methods for managing microbial processes in the engineered barriers

Factor	Management method	Landfill		SNF repository	
		Microbial process and level of effect	Environmental impact	Microbial process and level of effect	Environmental impact
Transport / supply of microbial substrates (CH_4, O_2, organic matter, SO_4)	Decreasing density of the material / increasing porosity	MO: + + +	CH_4 emission inhibition	SR: + +	Sulfide formation possible
Organic matter content	Decreased by material selection (soil / bentonite type)	MO: + HA: – –	CH_4 emission inhibition	SR: – – MP and HA: – – –	Minimized sulfide and CH_4 formation
Nutrient / mineral content	Increased by material selection, fertilization	MO: + / –	CH_4 emission inhibition / emissions possible	SR: + / –	Sulfide formation possible / inhibition of sulfide formation
Indigenous microbial population	Increased by material selection	MO: + + +	CH_4 emission inhibition	SR: + +	Sulfide formation possible

SNF, spent nuclear fuel; MO, methane-oxidation; HA, heterotrophic activity; SR, sulfate reduction; MP, methane production; OM, organic matter; n.m., not measured.

Organic matter content of the barrier material was found to have both indirect and direct effects on the activity of MOB and SRM, hence, providing one crucial factor to be considered in managing the engineered barriers. For the material used in the landfill covers, the natural organic matter does not directly serve as a substrate of the methanotrophs, which use methane as a source of carbon and energy (Khlemenina et al., 2018), but the high organic matter content was shown to be associated with both the activity of heterotrophic microorganisms and high WHC affecting the gas transport. The activity of heterotrophs and high WHC again were factors that affected positively the activity of MOB (papers I and II). The SRM and other microorganisms (e.g. methanogens) instead were presumed to benefit directly from the organic matter provided by the bentonites (paper III). However, the organic matter of different bentonites seemed to differ in the ability to sustain microbial growth as, for example, the organic matter of Bulgarian bentonite induced the highest microbial activity of all bentonites, which could be speculated based on the overall microbial activity (ATP) measured from the sand layers of the cells at the end of the cell experiment (Table 10; paper III). Therefore, the activity of the microorganism in the engineered barriers could be controlled via organic matter content of the materials, which again can be affected by material selection (Table 13).

The third main factor found to govern the activity of the MOB and SRM was the availability of inorganic compounds in the materials, which could be engineered by fertilization (nutrients) or by material selection (mineral composition of the bentonites) (Table 13). For MOB, the effect of fertilization on the activity was shown to be somewhat unpredictable as the attempts to increase the activity of MOB in the landfill cover soil by fertilization produced both increasing (trace elements and P in a batch assay at 4 °C, mixture of N, P, S, Cu in a column experiment at 21±2 °C) and decreasing (N in a batch assay at 4 °C; N, P and trace elements in a batch assay at 12 °C) responses in the activity of MOB (Figs. 11 and 12). The response of MOB to fertilization was possibly dependent on other prevailing factors, such as temperature or the composition of the methanotrophic community (paper II; Veraart et al., 2015). However, the inhibiting effect of the overall ionic content of the fertilized samples seemed to overcome the possible enhancing effect of the fertilization on the activity of MOB, which indicates that the ambient quantity and quality of the ion content of soil should also be considered in predicting the effect of possible fertilization (paper I). Similarly as in the landfill soil samples, the mineral composition and quantity of ions released from the bentonites by dissolution was

shown to affect the activity of the SRM; the low ambient gypsum content of the Bulgarian bentonite limited the availability of sulfate in the sand layer of the inoculated cell and, therefore, presumably the activity of SRM (Fig. 14f). Moreover, the high share of Fe^{3+} relative to the total iron content of the Indian bentonite (Table 4) elevated the redox potential of the sand layer solutions in both cells (>200 mV), which created conditions unbeneficial for the added or indigenous SRM in the sand layers of the cells (paper III).

Lastly, the indigenous microbial community of the soil and bentonite materials was found to partially contribute to the level of microbial activity in systems containing the materials. The quantity of indigenous MOB in the compost increased notably the number of MOB in the soil-compost mixture, which on its part enhanced the methane EC of the ameliorated soil (Table 9; Fig. 12b). As for the bentonites, the indigenous SRM community of the Wyoming and Bulgarian bentonites became active and spread from the bentonite blocks to the sand layers during the experiment (paper III; Table 10). In Indian and Wyoming bentonites instead, the indigenous SRM proliferated within the compacted bentonite blocks during the cell experiment (Table 11). Consequently, by controlling the number of indigenous microorganisms introduced to the engineered barriers within the materials, the performance of the barrier could be increased (methanotrophs) or possibly decreased (SRM) by material selection (Table 13). In practice, however, selection of the bentonite based on its indigenous microbial community is not feasible since other characteristics (e.g. smectite content) direct the selection of the material (Juvankoski, 2013).

6 CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

The main objective of this thesis was to assess the effect of different soils used in landfill covers on methane oxidation and the effect of bentonites that could be used as buffer or backfill in the SNF repositories on sulfate reduction. A further objective was to identify factors by which these microbial processes could be either enhanced (methane oxidation) or minimized (sulfate reduction) in the engineered barriers to provide information for managing the environmental impacts of disposal of MSW (methane emissions) and SNF (release of radionuclide due to sulfide-induced corrosion of copper).

The results of this thesis showed that the different soil and bentonite materials of the existing or planned release barriers can support naturally a vast range of microbial activities including methane oxidation and sulfate reduction. Moreover, the results showed that these microbial processes can be significantly affected by the chemical, physical, and/or microbiological properties of the soil and bentonite materials used in the structures. The results can be used as indicative examples of the expected processes and possibly utilized in managing and planning the release barriers even though the timescale of the conducted experiments (from days to 15 months) is rather short when compared to the operational times of the *in situ* applications (100 years for landfills, 1 000 000 year assessment period for the SNF repository). The aftercare of existing final covers in landfills could be beneficial for methane emission control, and the effect of the repository conditions on the fate of bentonite sOM should be considered when assessing the role of the introduced bentonite material as a source of substrates for sulfate reduction.

Both inorganic and organic soils of permanent and intermediate landfill cover structures appear to support methane oxidation activity as demonstrated with the 12 screened soil samples from two landfills (Tarastenjärvi, Tampere and Ämmäs-suo, Espoo, Finland). The magnitude of the measured methane oxidation activities was in accordance with the intended purpose of the landfill cover structures: the activities were 10x higher in the compost-based soils of the intermediate covers designed for use as release barriers during active filling phase of the landfill than in the mineral soils of the final covers designed more for landscaping purposes than for supporting

MOB activity (paper I). For emission control, the characteristics of the mineral landfill soil covers might need to be improved to support MOB to fully eliminate a typical methane load of a MSW landfill as demonstrated in the column experiment (paper II).

Several factors affecting the methane oxidation activity in landfill cover soils were identified. The compost-materials supporting the highest methane oxidation activities had high air-capacity due to higher organic matter content, WHC and porosity, which facilitate the diffusion of oxygen and methane in the soil material (papers I, II). For these reasons, compost addition (22 w-%) was the most efficient method for improving the MOB activity of the mineral landfill soil (paper II).

The MOB activity appears to be dependent on the nutrient and in particular nitrate content of the soil material, which is higher in compost materials than in mineral soils (papers I, II). However, fertilization of the soil with different inorganic nutrients (N, P, and trace elements) did not induce a long-lasting increase in the MOB activity of the mineral landfill soil (paper II). The significance of trace elements for the MOB activity was highlighted only at low temperature (4 °C) (paper I).

The quantity and presumably quality of the organic matter in the bentonites can affect the activity of microorganisms (e.g., SRM, methanogens, presumably IRB). The Bulgarian bentonite induced a higher overall microbial activity than the Wyoming and Indian bentonites based on the ATP concentrations measured after the experiment (paper III). The Bulgarian bentonite was also shown to contain the highest amount of sOM, followed by the Wyoming and Indian bentonites (163, 85 and 16 mg kg^{-1}, 12, 4 and 2% of TOC, respectively) (paper IV).

The mineralogy of the bentonites affected the microbial activity. The low gypsum content of the bentonite could limit the activity of SRM and, thus, production of sulfide as demonstrated by the low SRM activity and depletion of sulfate from the sand layer of the inoculated cell of the Bulgarian bentonite (paper III). The low ratio of Fe^{2+} to F^{3+} in the Indian bentonite increased the redox potential of the sand layer solution, which was believed to suppress the activity of SRM at least in the sand layers of the cells (paper III).

The simulated repository conditions, compaction to target dry density of 1400 kg m^{-3} and microbial activity in the sand layer (the simulated interface of bentonite and bedrock) and/or within the bentonite, were shown to increase the bentonite sOM quantity and release rate compared to the original bentonites up to 20% of TOC (in the Bulgarian bentonite) (paper IV). However, the possible effect of increase in this microbially available bentonite organic matter fraction on the microbial activity could not be detected during the cell experiment, which suggests that the release of

sOM from the bentonites is controlled by factors (e.g. diffusion) not studied in this thesis.

Further studies on landfill covers could focus on the effect of nitrate (and other nutrients) addition on MOB activity as their impact appears to be a complex process and dependent on other simultaneously prevailing factors. If the process was understood better, the composition of fertilizers could be planned more carefully, and fertilization could possibly be used as a method for improving the MOB activity in nutrient-deprived soils of old landfill covers. Even though the effect of different nutrients on metabolism and growth of the MOB has been studied in this thesis and also by many other researchers (e.g. Zheng et al., 2013; Veraart et al., 2015), no unambiguous explanation for MOB's response to the additions of different nutrients has been found. Here, the nitrate content of the soil was identified as one of the significant factors predicting the MOB activity by the MLR analysis, but the response of the MOB activity to the nitrate addition in the batch assays conducted at 4 and 12 °C and in the column experiment was both a decrease and increase, respectively.

Another issue requiring further study is the temporal significance of the bentonite as a source of substrate for the microorganisms, which is linked to the bioavailability of the organic matter. The results of this thesis showed that microorganisms can utilize bentonite organic matter but it is possible that, in terms of the time scale the EBS is expected to provide passive safety, the bentonite organic matter might not have a significant role in driving microbial activity if the bioavailability of the sOM fraction was low. The bioavailability of the sOM could not be determined from the data obtained in the present cell experiment, but stabilization of the DOC concentrations in the sand layers of the cells (2–23 mg L^{-1} on average), suggests that a diffusive equilibrium was attained in the sand layer solution with the DOC concentration of the bentonite porewaters and, thus, some fraction of the organic matter could not be consumed by the microorganisms (paper IV; Kiczka et al., 2021). Quantification of the bioavailable fraction of sOM would help in assessing the amount of sulfide produced by the SRM when consuming bentonite sOM in the actual repository.

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PUBLICATIONS

PUBLICATION
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Methane oxidation potential of boreal landfill cover materials: The governing factors and enhancement by nutrient manipulation

Maanoja, S. & Rintala, J.

Methane oxidation potential of boreal landfill cover materials: The governing factors and enhancement by nutrient manipulation

CrossMark

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ABSTRACT

Methanotrophs inhabiting landfill covers are in a crucial role in mitigating CH_4 emissions, but the characteristics of the cover material or ambient temperature do not always enable the maximal CH_4 oxidation potential (MOP). This study aimed at identifying the factors governing MOPs of different materials used for constructing biocovers and other cover structures. We also tested whether the activity of methanotrophs could be enhanced at cold temperature (4 and 12 °C) by improving the nutrient content (NO_3^-, PO_4^{3-}, trace elements) of the cover material. Compost samples from biocovers designed to support CH_4 oxidation were exhibiting the highest MOPs (4.16 µmol CH_4 g_{dw}^{-1} h^{-1}), but also the soil samples collected from other cover structures were oxidising CH_4 (0.41 µmol CH_4 g_{dw}^{-1} h^{-1}). The best predictors for the MOPs were the NO_3^- content and activity of heterotrophic bacteria at 72.8%, which were higher in the compost samples than in the soil samples. The depletion of NO_3^- from the landfill cover material limiting the activity of methanotrophs could not be confirmed by the nutrient manipulation assay at 4 °C as the addition of nitrogen decreased the MOPs from 0.090 µmol CH_4 g_{dw}^{-1} h^{-1} to <0.085 µmol CH_4 g_{dw}^{-1} h^{-1}. At 12 °C, all nutrient additions reduced the MOPs. The inhibition was believed to result from high ionic concentration caused by nutrient addition. At 4 °C, the addition of trace elements increased the MOPs (>0.096 µmol CH_4 g_{dw}^{-1} h^{-1}) suggesting that this was attributable to stimulation of the enzymatic activity of the psychrotolerant methanotrophs.

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1. Introduction

Methane (CH_4), with current atmospheric concentration of 1.8 ppm, is recognised as one of the most effective greenhouse gases contributing to the climate warming due to its high potential for heat absorption (Ciais et al., 2013; Lelieveld et al., 1998). Waste treatment sector is the third largest anthropogenic source of global CH_4 emissions (Ciais et al., 2013), and approximately 82% of the emissions of this sector are originating from landfilling of solid wastes in the countries of European Union (4.8 Mt CH_4 yr^{-1} in 2012) (EEA, 2014). Several actions have been taken to mitigate CH_4 emissions from landfills, including collection, valorisation and flaring of the gas. However, these techniques are not feasible or economical in ageing, minor or remote landfills, and in those cases the top soil covers of the landfills are in a crucial role in mitigating CH_4 emissions (Huber-Humer et al., 2008). These soil covers contain methanotrophs, naturally occurring bacteria, that utilise CH_4 as a sole source of carbon and energy and oxidise it to

carbon dioxide (CO_2), water and new biomass (Hanson and Hanson, 1996).

CH_4 oxidation potential (MOP) of landfill cover material is affected by several factors, including pH, moisture and organic matter contents of the soil, nutrient availability (nitrogen, phosphorus) and physical properties enabling gas diffusion (Einola et al., 2007; Huber-Humer et al., 2011, 2009; Röwer et al., 2011; Wang et al., 2011). When the cover structure is particularly designed for supporting CH_4 oxidation, such as intermediate covers of active landfills and final covers of closed landfills, some of the parameters can be optimised for methanotrophs via material selection. For example, different types of organic-rich compost material are frequently utilised for the biocovers as they possess naturally high MOP, and their CH_4 removal efficiency might reach 100% (Huber-Humer et al., 2008; Scheutz et al., 2009). Furthermore, landfill covers consist of different types of mineral soil material as part of the infrastructure not especially designed for supporting CH_4 oxidation. Due to the large quantity of soil required for closing and landscaping the landfill surface, the material is often selected based on the availability and cost. Thus, they might include soils with characteristics, such as fine texture and low organic matter content, not suitable for supporting high MOP (Bohn et al., 2011).

In soil covers, the CH$_4$ removal efficiency might remain at 40–45% while the rest emits to the atmosphere (Scheutz et al., 2009).

In addition to the characteristics of the cover material, one significant factor affecting the activity of methanotrophs in landfill covers is temperature. The optimum temperature for methanotrophs is 20–35 °C (Spokas and Bogner, 2011) and below that the activity of the CH$_4$ mono-oxygenase enzyme (MMO) catalysing the first reaction of CH$_4$ oxidation decreases and the MOP of the material might fall even to 23% of the initial value (Einola et al., 2008, 2007). Furthermore, earlier studies have shown that methanotrophs are more sensitive to other regulatory factors, such as nutrient limitation, when the ambient temperature is below their optimum range (<15 °C) (Albanna and Fernandes, 2009). The nutrient content of the cover materials decreases naturally in the course of time due to plant growth and microbial processes (Scheutz et al., 2009) and, thus, might magnify the negative effect of the low temperature on methanotrophs. This could potentially be prevented by adding nutrients to the cover material; MOP of a biocover soil was increased by 144% at 5 °C after addition of NPK-fertilizer in batch assays (Albanna and Fernandes, 2009). As for trace elements, the psychrotolerant type I methanotrophs have a higher demand for copper than mesophilic type II methanotrophs (Börjesson et al., 2004; Hanson and Hanson, 1996) and could benefit from copper addition.

The aim of this study was to screen the MOPs of different types of landfill cover material collected from two landfills and to analyse the factors governing their MOPs. The samples included materials from engineered biocovers (compost) and from other landfill cover structures not particularly intended for supporting CH$_4$ oxidation (soil, sand), but which might become exposed to CH$_4$ due to fissures in the sealing layers or leakages in the gas collection system. The attention was focused on the physical characteristics and nutrient content of the samples. In addition, one compost sample was used to assess the potential of nutrient addition (nitrogen, phosphorus, trace elements) to improve the MOP at 4 and 12 °C, which are temperatures outside of methanotrophs' optimum temperature range (Wang et al., 2011). Maintaining the activity of the methanotrophs also at low temperature is critical for emission control.

2. Materials and methods

2.1. Sample collection

In total twelve soil samples were collected from Ämmässuo and Tarastenjärvi landfills located, respectively, in Espoo (60.2°N, 24.5° E) and Tampere (61.2°N, 23.5°E), Finland. The landfills chosen for the study represented two different types of landfills with different cover structures, one in an operation phase (Ämmässuo) and one in a post-closure phase (Tarastenjärvi).

The sub-area of Ämmässuo landfill, 12.6 ha in area, has been in operation since 2007 and has received approximately 375,000 tons of municipal, construction and industrial waste per year. Six samples were collected from two depths (10–20 and 40–50 cm) of two biocovers distributed in 2007 (As1 and As2) and from one biocover distributed in 2010 (As3) (Fig. 1). The total depth of the intermediate biocover was 50 cm and it consisted of a mixture of compost and fine stone aggregates (80:20 v/v) with clay (As3 10–20, 10% v/v) and sand (As3 40–50, 20% v/v). Samples were collected in November 2012 when the ambient air temperature was 0.8 °C and the soil temperature ranged from 5.8 to 12.5 °C.

Tarastenjärvi landfill was established in 1977 and has since received 3.4 million tons of municipal solid waste and industrial and construction waste on land area of 30 ha. Six locations were chosen from different parts of the landfill to represent different soil
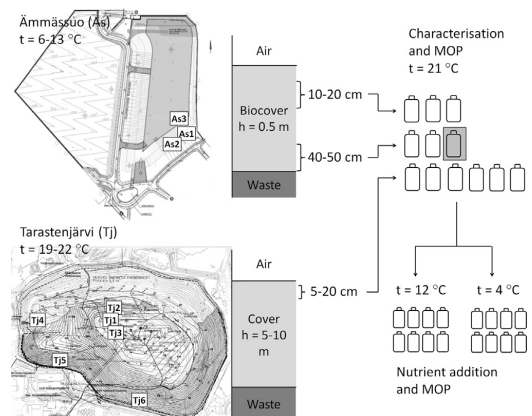
Fig. 1. The sampling locations in Ämmässuo (As1–3) and Tarastenjärvi (Tj1–6) landfills (60.2°N, 24.5°E and 61.2°N, 23.5°E, respectively), sampling depths and the experimental design. One sample from Ämmässuo was chosen for further experiments due to its low CH$_4$ oxidation potential (MOP) and nutrient content.

types (Fig. 1); two samples from the top of the old backfill of waste (Tj1 and Tj2; final cover soil, distributed in 2007), one from the foundation of a new gas well on top of the old backfill of waste (Tj3; sand, constructed in 2007), two from areas sealed in the 1990s (Tj4 and Tj5; final cover soil, Tj5 with loam, Tj4 reported to emit CH$_4$) and one from the area being under landscaping (Tj6; compost-based mixture, distributed in 2012). Samples were collected from one pooled depth (5–20 cm) in July (Tj1–3) and August 2013 (Tj4–6). The temperature of air ranged from 18.5 °C to 22.0 °C. The soil temperature was not recorded, but it has been reported from another Finnish landfill that the temperature of final cover top soil (5–20 cm; ~23 °C) corresponds approximately to the ambient temperature (25.1 °C) in summer time (June) (Einola et al., 2009).

At each sampling site, approximately 5 L of soil was transferred into plastic buckets and stored at 4 °C maximum for 5 days prior to chemical analyses.

2.2. Determination of MOP

MOPs of the samples were determined by batch assays. The cover material sample was sieved and 8–12 g of the fraction of <2 mm was incubated in a 120 mL glass bottle with 7% CH$_4$ (v/v; Aga Ltd. Finland) in the headspace. The initial concentration of 7% CH$_4$ corresponded to the range of 5–10% CH$_4$ (v/v) often used in CH$_4$ oxidation experiments of landfill soil (Albanna and Fernandes, 2009; Einola et al., 2007; Röwer et al., 2011; Wang et al., 2011). The ratio of O$_2$/CH$_4$ in the incubation bottles was approximately 4:1, which was sufficient to support complete aerobic oxidation of CH$_4$ and respiratory activities of heterotrophic bacteria (Chi et al., 2012). The assays for basal respiration (BR) of the soil samples were prepared similarly, but no CH$_4$ was inserted to the bottles in order to measure the respiration of other than methanotrophic bacteria i.e. the heterotrophic bacteria (Einola et al., 2007).

Samples were incubated at room temperature (~21 °C) covered from light. Sub-samples of the gas phase were analysed regularly for CH$_4$ and CO$_2$ until the concentration of CH$_4$ was lower than the detection limit of the gas chromatograph (<0.1% v/v), and then the experiment was finished. Depending on the activity of the bacteria in each sample, the duration of the incubation was 23–360 h.

Samples from Ämmässuo landfill were pre-incubated with CH_4 to induce the CH_4 oxidation activity prior to the actual assay after long storage time of the samples (5 weeks). The storage time was long due to practical reasons as the incubation setup was optimised and preliminary tests, such as determination of moisture content, were carried out prior to the determination of MOP. It has been shown earlier that the CH_4 oxidation activity of the soil methanotrophs remains unaltered after storing of 20 weeks at 4 °C (Einola et al., 2007). Samples from Tarastenjärvi were not pre-incubated as their storage time was shorter (0–4 d).

All Ämmässuo samples were incubated in the ambient moisture content (31% of wet weight (ww) on average), which was within the optimal range of moisture content of methanotrophs (30–50%$_{ww}$) (Wang et al., 2011). The moisture content of samples Tj1–3 and Tj5 was substantially below the optimal range of methanotrophs and the samples did not oxidise CH_4 in the initial batch assay carried out in ambient moisture content (data not shown). Thus, the moisture content was adjusted to 33%$_{ww}$ by adding de-ionised H_2O. This corresponded to 46% of the average water holding capacity (WHC) of the Tarastenjärvi samples, which was estimated from the WHCs of samples Tj4 and Tj6 determined according to FCQACO (1994) (79 and 65% of dry weight (dw), respectively). Moisture content corresponding approximately to 50% of the WHC of material is considered optimal for methanotrophs (Huber-Humer et al., 2009).

2.3. Effect of nutrient manipulations on MOP

To study whether the nutrient addition could be used to increase the MOP at 4 and 12 °C, the compost sample As2 40–50 was chosen for the assay as the sample contained less NO_3^- and PO_4^{3-} than the other samples. In addition, As2 40–50 exhibited a lower MOP than most of the Ämmässuo samples, so it was assumed that the increase in the MOP could be easily detectable in this sample.

The study on effect of nitrogen (N), phosphorous (P) or trace elements (Te) (Cu, Fe, Zn, Mn, Ni, Mo) addition on MOP was created using a full factorial, balanced and randomized design with one level of three factors. Thus, the samples received a single-addition (N, P, Te), a dual-addition (N + P, N + Te, P + Te) or a triple-addition (N + P + Te) of nutrients. The composition of the three nutrient solutions (N, P, Te) was as follows per litre of distilled water: solution N: 31.1 g KNO_3, 24.7 g $MgSO_4$·$7H_2O$, 4.6 $CaCl_2$; solution P: 14.6 g Na_2HPO_4, 26.8 g KH_2PO_4; solution Te: 4.7 mg $CuSO_4$·$5H_2O$, 11.5 mg $FeSO_4$·$7H_2O$, 9.2 mg $ZnSO_4$·$7H_2O$, 6.3 mg EDTA-Na_2·$2H_2O$, 0.45 mg $MnCl_2$·$4H_2O$, 0.30 $NiSO_4$·$6H_2O$, 5.9 mg Na_2MoO_4·$2H_2O$.

Each soil sample (10–12 g$_{ww}$) was sprayed with 270 µl of nutrient solution (control samples with H_2O) containing the targeted amount of one or more nutrients. Nutrient addition increased moisture content of the samples from 26.5%$_{ww}$ to 28.0%$_{ww}$. Samples were injected with CH_4 and air for measuring the MOP and BR as described earlier. One set of samples was incubated at 4 °C and the other at 12 °C.

After additions the computational nutrient contents of the samples were on average 650 mg NO_3^- kg$_{dw}^{-1}$ (N), 960 mg PO_4^{3-} kg$_{dw}^{-1}$ (P) and 0.040 mg Cu^{2+} kg$_{dw}^{-1}$ (Te) (initial + added concentration) where applied (Table 1). The concentrations for nutrients were chosen based on the literature on stimulation of methanotrophic activity: the MOP of soil has been increased significantly with additions up to 600 mg NO_3^- kg$_{dw}^{-1}$ (Wang et al., 2011). The PO_4^{3-} concentration was adjusted to correspond the recommended mass ratio of N/P $\geqslant$ 0.5 for methanotrophs' growth medium (Nikiema et al., 2013). The concentration of Cu^{2+} was adjusted to be high enough to induce expression of MMOs in type I methanotrophs but not

Table 1
Amount of soluble nutrients in the sample (As2 40–50, m = 10–12 g$_{ww}$) before and after nutrient additions in the incubation experiment conducted at two temperatures, 4 and 12 °C (dw = dry weight; ww = wet weight; MC = moisture content; n.m. = not measured).

Ion (mg kg$_{dw}^{-1}$)	Before	After	
		4 °C	12 °C
NO_3^-	14.1	600	700
PO_4^{3-}	BD[a]	870	1040
Cu^{2+}	n.m.	0.036	0.043
N/P (mg:mg)	–[b]	0.51	0.50
MC (%$_{ww}$)	26.5	28.0	28.0

[a] BD, below limit of detection (<2 mg kg$_{dw}^{-1}$).
[b] Initial ratio of N/P could not be calculated as the accurate concentration of PO_4^{3-} could not be determined.

too high to inhibit the expression of MMOs in type II methanotrophs (Van der Ha et al., 2013).

2.4. Analytical methods and calculations

Moisture content and dry weight of the sample were determined according to the standard SFS-EN 14346 by drying 5–30 g wet soil at 105 °C. Organic matter content of the soil sample was determined as loss on ignition at 550 °C following the standard SFS-EN 15169. Results were not corrected for carbonates which might start decomposing at temperatures <550 °C (Santisteban et al., 2004). pH of the soil samples was determined from a suspension of air-dried (<40 °C) soil and 0.01 M $CaCl_2$ (1:5, v/v) according to the standard SFS-ISO 10390. Electrical conductivity of the air-dried samples was determined following the standard CEN/TS 15937 from an unfiltered suspension of air-dried soil and de-ionised H_2O (1:2.5, v/v).

Changes in the concentrations of CH_4 and CO_2 in the bottles were monitored by analysing sub-samples of the headspace with a gas chromatograph coupled with a thermal conductivity detector (Shimadzu GC-2014). The column used was Agilent 80/100 Porapak N and the temperatures of the injector, oven and detector were 80 °C, 40 °C and 80 °C, respectively, and the flow rate of carrier gas 25 mL He min^{-1}. Samples were analysed against a known standard gas CH_4:CO_2, 50:50 (v/v) (Aga Ltd., Finland).

For calculating the MOPs and CO_2 production rates, the measured gas concentrations were plotted as a function of time and the initial linear parts of the graphs (10–310 h) were used to determine the slopes. The amount of CO_2 produced by methanotrophs was calculated by subtracting the amount of CO_2 produced in the BR samples (injected with air) from the amount of CO_2 produced in the MOP samples (injected with CH_4). The carbon assimilation efficiency of methanotrophs was calculated from the mass balance of carbon originating from the amount of oxidised CH_4 – it was assumed that the remaining fraction x not measured as amount of CO_2 had been assimilated to the biomass of methanotrophs according to Eq. (1) (Urmann et al., 2007):

$$CH_4 + (2 - x)O_2 \rightarrow (1 - x)CO_2 + xCH_2O + (2 - x)H_2O \qquad (1)$$
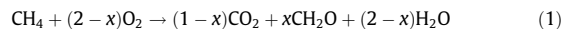
The concentrations of nutrients (NO_3^-, PO_4^{3-}, SO_4^{2-}) were analysed from a water extract of soil samples (soil:water 1:4, w/v) using an ion chromatograph (Dionex ICS-1600) with conductivity detection. The eluent consisted of 1.9 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ and the flow rate was 1.0 mL min^{-1}. The temperatures of the cell and column ovens (Dionex IonPac AS4A-SC 4 × 250 mm) were 35 and 30 °C, respectively.

2.5. Statistical analyses

The multiple linear regression (MLR) analysis was performed on the data to test whether the different cover material characteristics

could be used to predict the MOP. Forward stepwise selection of variables was used to address the best predictors for MOP. Only one variable from each correlated pair ($r < |0.7|$) was included to the MLR analysis to avoid multicollinearity of the variables; MOP of the soil as a dependent variable and content of NO_3^- and organic matter of the soil, pH and BR rate as independent variables. Content of SO_4^{2-} and electrical conductivity were excluded from the analysis as they had a poor correlation with MOP as their own ($r < 0.3$). In addition, moisture content was excluded as it was manipulated for some batch assay samples and content of PO_4^{3-} was excluded due to missing data.

The effect of nutrient additions on MOP and BR rates was tested separately for both incubation temperatures (4, 12 °C). The mean values were compared with Student's 2-tailed *t*-test with confidence level set at 95%. The critical *t*-value at two degrees of freedom is 4.303.

3. Results

3.1. Characteristics of the landfill cover material governing the MOPs

All the 12 samples (Ämmässuo, three locations, two depths; Tarastenjärvi, six locations, one depth) were capable of oxidising CH_4 during batch assays at 21 °C (Table 2). The MOPs of Ämmässuo compost samples were on average 4.16 ± 0.764 SE μmol CH_4 g_{dw}^{-1} h^{-1} ($n = 6$) of which four ranged between 4.44 and 5.98 μmol CH_4 g_{dw}^{-1} h^{-1} while the two samples collected from the deeper sampling depth were ⩽2.1 μmol CH_4 g_{dw}^{-1} h^{-1} (As2 40–50, As3 40–50). The MOPs of five Tarastenjärvi samples were on average 0.41 ± 0.151 SE μmol CH_4 g_{dw}^{-1} h^{-1} (excluding Tj4). The exception to low potentials at Tarastenjärvi was the MOP measured from one of the final cover soil samples (Tj4: 7.21 μmol CH_4 g_{dw}^{-1} h^{-1}) and it was in the same order of magnitude as the MOPs of Ämmässuo landfill. The carbon assimilation efficiencies of the methanotrophic bacteria ranged from 48% to 74% between samples (Table 2).

The BR was highest in samples containing compost, on average 0.54 μmol CO_2 g_{dw}^{-1} h^{-1} ($n = 4$, excluding As2 10–20, As3 40–50).

One compost sample collected from the shallower sampling depth had a significantly higher BR rate than the average value (As2 10–20; 2.01 μmol CO_2 g_{dw}^{-1} h^{-1}), while one collected from the deeper sampling depth showed rather low heterotrophic activity, 0.05 μmol CO_2 g_{dw}^{-1} h^{-1} (As3 40–50). Three Tarastenjärvi samples consisting of soil had a BR rate of 0.13 μmol CO_2 g_{dw}^{-1} h^{-1} on average (excluding Tj4). One soil sample collected from the final cover soil in Tarastenjärvi (Tj4) had a slightly higher BR rate than the others, 0.50 μmol CO_2 g_{dw}^{-1} h^{-1}. The lowest heterotrophic activity among all samples was measured from the sand sample of Tarastenjärvi, 0.04 μmol CO_2 g_{dw}^{-1} h^{-1} (Tj3).

Chemical and physical characteristics of the 12 soil samples were determined (Table 2). pH of the soil was close to neutral (6.9–8.1) in all samples except in two samples containing loam, which were slightly acidic (4.8–5). The one sample of loamy compost and five samples of compost had higher moisture content (22–52%$_{ww}$) than the rest six samples containing sand and soil (6–23%$_{ww}$). The loamy compost sample also had the highest organic matter content (As3 10–20; 28%$_{dw}$), whereas the lowest contents were measured from the three samples containing sand or loamy soil (3–5%$_{dw}$). The organic matter content of the other five compost samples varied between 5 and 22%$_{dw}$.

All samples from Tarastenjärvi and two of the compost samples of Ämmässuo (As2) contained ⩽52 mg NO_3^- kg_{dw}^{-1}, whereas the other four compost samples of Ämmässuo contained 167–358 mg NO_3^- kg_{dw}^{-1} (Table 2). The concentration of PO_4^{3-} could not be determined from several samples as it was below the limit of detection of the analytical instrument (<2 mg kg_{dw}^{-1}). The highest concentration of PO_4^{3-}, 52 mg kg_{dw}^{-1}, was measured from the top depth of one of the compost biocovers of Ämmässuo (As2 10–20). The two samples containing compost and two soil samples contained 5.1–15 mg PO_4^{3-} kg_{dw}^{-1}. The concentration of SO_4^{2-} was lower in the four soil samples (<80 mg kg_{dw}^{-1}) than in the seven compost samples (125–8500 mg kg_{dw}^{-1}) or in the sand sample (1230 mg kg_{dw}^{-1}). The electrical conductivity of the 12 samples ranged from 52 to 2787 μS cm^{-1} and correlated strongly with the total anion content of the soil samples ($r = 0.96$, $p < 0.001$).

The MLR analysis was used to test statistically the predictability of MOP by different soil characteristics in all 12 soil samples. The

Table 2

Characteristics of landfill biocover soil samples collected from Ämmässuo (As) and Tarastenjärvi (Tj) landfill and CH_4 oxidation potentials (MOPs, μmol g_{dw}^{-1} h^{-1}) and CO_2 production rates (Prod$_{CO2}$, μmol g_{dw}^{-1} h^{-1}) measured during batch assays from CH_4 oxidation and basal respiration (BR) samples (Y_c = carbon assimilation efficiency of methanotrophs, EC = electrical conductivity, MC = moisture content, ww = wet weight, OM = organic matter content, dw = dry weight, BD = below limit of detection (<2 mg kg_{dw}^{-1})).

Sampling location[a]	Distributed[b] Year	Soil type[c]	MOP[d] by CH_4 oxidation	Prod$_{CO2}$[d] via BR	Prod$_{CO2}$[d] by methanotrophs (%)	Y_c (%)	pH	EC (μS cm^{-1})	MC[e] (%$_{ww}$)	OM (%$_{dw}$)	NO_3^-	PO_4^{3-}	SO_4^{2-} (mg kg_{dw}^{-1})
As1 10–20	2007	Compost	5.68	2.85	0.41	85	57	7.4	559	30	13	358	BD	125
As1 40–50	2007	Compost	5.17	2.51	0.34	86	58	6.9	496	22	5	232	BD	251
As2 10–20	2007	Compost	4.44	3.99	2.01	49	55	7.4	2787	38	22	18	52	7690
As2 40–50	2007	Compost	2.13	1.50	0.40	72	48	7.4	2020	31	14	14	BD	8519
As3 10–20	2010	Loamy compost	5.98	3.49	0.91	74	57	5.0	923	52	28	167	5.1	1393
As3 40–50	2010	Sandy compost	1.58	0.61	0.05	91	64	7.7	552	15	3	184	BD	449
Tj1	2007	Soil	0.20	0.16	0.09	45	64	7.3	291	16	6	23	BD	78
Tj2	2007	Soil	0.27	0.24	0.13	47	58	7.0	192	9	9	24	12	42
Tj3	2007	Sand	0.08	0.07	0.04	33	74	8.1	1176	6	3	52	BD	1234
Tj4	In 1990s	Soil	7.21	4.02	0.50	77	51	5.8	160	23	12	22	15	14
Tj5	In 1990s	Loamy soil	0.59	0.46	0.19	58	55	4.8	52	11	5	14	BD	14
Tj6	2012	Compost	0.91	0.97	0.63	35	63	7.0	1061	23	11	15	6.8	802

The values are means of duplicate (MOP, Prod$_{CO2}$), triplicate (pH, EC) and four parallel samples (MC, OM, nutrients).

[a] Samples from each location of As were collected from two depth layers, 10–20 cm and 40–50 cm and for Tj from one pooled depth 5–20 cm.

[b] Samples were collected from Ämmässuo in 2012 and from Tarastenjärvi in 2013.

[c] Soil type categorised as "loamy compost" consisted of compost (70%), fine stone aggregates (20%) and clay (10%); "sandy compost" consisted of compost (60%) and fine stone aggregates (40%); "loamy soil" consisted of soil (70%) and clay (30%) (v/v).

[d] The standard error relative to mean (RSE) is ⩽10% for MOPs, <8% for CO_2 production in MOP samples and <5% (except for As1 10–20, As1 40–50 and Tj3, where RSE is 11–18%) for CO_2 production in basal respiration samples.

[e] The moisture content of samples Tj1–3 and Tj5 was increased from the ambient to 33%$_{ww}$ for determination of MOP.

variance in the MOP data was best explained at 70.1% by the BR and NO_3^- content of the soil when all variables were included (Table 3a). The contribution of organic matter and pH was insignificant ($p > 0.05$). The MLR model including only the significant variables explained 72.8% of the variance in the MOP data, with BR contributing positively and NO_3^- content negatively (Table 3b). The NO_3^- data were transformed prior to analysis so the contribution of untransformed data on the MOPs was positive.

3.2. The effect of nutrient additions on MOPs and BRs at 4 and 12 °C

The hypothesis for the nutrient manipulation assay was that by relieving nutrient limitation of the methanotrophs (N, P, Te) in temperatures below their optimal range (4 and 12 °C) the MOP of the nutrient-depleted compost sample (As2 40–50) would increase. Resulting from the nutrient additions at 4 °C, all samples treated with N or a solution mixture containing N exhibited lower MOPs ($<0.085 \ \mu mol \ CH_4 \ g_{dw}^{-1} \ h^{-1}$) than the control sample ($0.090 \ \mu mol \ CH_4 \ g_{dw}^{-1} \ h^{-1}$), whilst samples treated with P and Te exhibited higher MOPs ($>0.096 \ \mu mol \ CH_4 \ g_{dw}^{-1} \ h^{-1}$) (Table 4, Fig. 2A). At 12 °C, CH_4 was oxidised slower in all treated samples ($<0.473 \ \mu mol \ CH_4 \ g_{dw}^{-1} \ h^{-1}$) than in the control sample ($0.499 \ \mu mol \ CH_4 \ g_{dw}^{-1} \ h^{-1}$), but the stimulation by P and Te and inhibition by N followed the same pattern as at 4 °C (Table 4, Fig. 2D). At 4 °C, only P + Te addition induced a significant difference in the MOP, when compared with the control sample ($t = 7.355$, $p < 0.05$). Between different treatments, CH_4 was oxidised significantly slower in samples with N + P and N + P + Te than

in samples with Te and P + Te (Fig. 2A). At 12 °C, the differences between MOPs were not significant (Fig. 2D).

At 4 °C, the addition of N and N + Te resulted in the lowest BR rates (0.040 and 0.039 $\mu mol \ CO_2 \ g_{dw}^{-1} \ h^{-1}$), whereas the rest of the additions induced an increase in the respiration rate ($>0.047 \ \mu mol \ CO_2 \ g_{dw}^{-1} \ h^{-1}$) compared to the control (0.042 $\mu mol \ CO_2 \ g_{dw}^{-1} \ h^{-1}$) (Table 4, Fig. 2C). At 12 °C, only the N + P addition stimulated slightly BR (0.168 $\mu mol \ CO_2 \ g_{dw}^{-1} \ h^{-1}$), while the other additions had a decreasing effect ($\leqslant 0.163 \ \mu mol \ CO_2 \ g_{dw}^{-1} \ h^{-1}$) compared to the control (0.163 $\mu mol \ CO_2 \ g_{dw}^{-1} \ h^{-1}$) (Fig. 2F).

At 4 °C, the addition of Te increased the carbon assimilation efficiency of methanotrophs to 0.45, twice as high as the control sample. Other nutrient additions did not induce as great increase in the assimilation efficiencies compared to the control, and they ranged from 0.21 to 0.36. The carbon assimilation efficiency in samples treated with Te was significantly higher than in samples treated with N and N + Te ($p < 0.05$, Fig. 3). At 12 °C, nutrient additions did not induce as notable increase in the carbon assimilation efficiencies compared to the control (0.50) and they ranged from 0.38 to 0.57. The sample treated with Te had significantly higher carbon assimilation efficiency than the samples treated with P + Te and N + P + Te (Fig. 3).

4. Discussion

The 12 studied soil samples, which were collected from two landfills and comprised of compost (As1–3, Tj6), soil (Tj1–2, 4–5) and sand (Tj3), were all found to oxidise CH_4 with notable rates.

Table 3

Multiple linear regression (MLR) of biocover soil characteristics and CH_4 oxidation potential (MOP). Standardised and unstandardised correlation coefficients R with standard error. Tolerance as a measure of multiple correlations between variables (dw = dry weight, BR = CO_2 production in basal respiration samples, OM = organic matter).

	Standardised R	Unstandardised R	SE	Tolerance	t	p
(a) All variables included. N = 12, multiple $R^2 = 0.810$, adjusted multiple $R^2 = 0.701$, $F_{4,7} = 7.462$, $p = 0.011$						
Intercept		3.226	0.807		3.999	0.005
NO_3^- (mg g_{dw}^{-1})	−0.609	−19.486	5.776	0.834	−3.374	0.012
BR ($\mu mol \ CO_2 \ g_{dw}^{-1} \ h^{-1}$)	0.985	1.685	0.532	0.281	3.167	0.016
OM ($\%_{dw}$)	−0.248	−0.028	0.034	0.307	−0.832	0.433
pH	0.146	0.683	0.875	0.781	0.781	0.461
(b) The best MLR model to predict variance in MOP data. N = 12, multiple $R^2 = 0.777$, adjusted multiple $R^2 = 0.728$, $F_{2,9} = 15.717$, $p < 0.001$						
Intercept		2.914	0.303		9.609	<0.001
NO_3^- (mg g_{dw}^{-1})	−0.547	−17.499	5.185	0.943	−3.375	0.008
BR ($\mu mol \ CO_2 \ g_{dw}^{-1} \ h^{-1}$)	0.835	1.429	0.277	0.943	5.154	0.001

Data were square root transformed (MOP), $\log_{10}$ transformed (BR), inversed (NO_3^-) or inversed before $\log_{10}$ transformation (pH) to meet the assumptions of the statistical analysis.

Table 4

CH_4 oxidation potentials (MOP) and CO_2 production (Prod$_{CO2}$) rates in CH_4 oxidation and basal respiration samples incubated at 4 and 12 °C after addition of nutrients (N = nitrogen, P = phosphorus, Te = trace elements) or water (Ctrl) (mean values, $n = 2$).

Temperature	Treatment	MOP ($\mu mol \ CH_4 \ g_{dw}^{-1} \ h^{-1}$) by CH_4 oxidation	Prod$_{CO2}$ ($\mu mol \ CO_2 \ g_{dw}^{-1} \ h^{-1}$) by CH_4 oxidation	Prod$_{CO2}$ ($\mu mol \ CO_2 \ g_{dw}^{-1} \ h^{-1}$) via basal respiration	Prod$_{CO2}$ by methanotrophs (%)
4 °C	Ctrl	0.090	0.110	0.042	62
	N	0.085	0.105	0.040	62
	P	0.096	0.112	0.049	57
	Te	0.104	0.112	0.056	50
	N + P	0.081	0.112	0.049	56
	N + Te	0.077	0.100	0.039	61
	P + Te	0.100	0.117	0.047	60
	N + P + Te	0.078	0.102	0.052	49
12 °C	Ctrl	0.499	0.416	0.163	60
	N	0.143	0.182	0.127	29
	P	0.473	0.385	0.139	64
	Te	0.406	0.337	0.163	50
	N + P	0.287	0.326	0.168	48
	N + Te	0.256	0.278	0.142	48
	P + Te	0.466	0.425	0.136	68
	N + P + Te	0.174	0.221	0.118	43
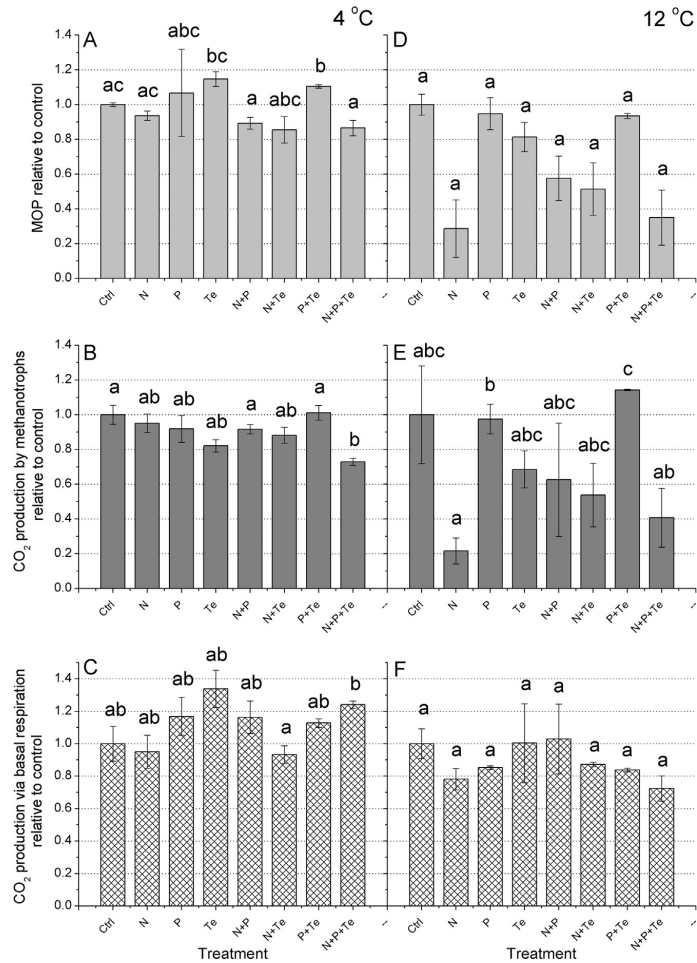
Fig. 2. CH_4 oxidation potentials (MOP) (A and D) and CO_2 production rates in CH_4 oxidation (B and E) and basal respiration samples (C and F) treated with nutrient solutions (N = nitrogen, P = phosphorus, Te = trace elements) relative to the untreated control sample (Ctrl) at 4 °C (A–C) and 12 °C (D–F) (mean values ± SE, $n = 2$). Different small alphabets indicate statistically significant difference between different treatments (Student's 2-tailed t-test, df = 2, MOP: 4 °C: $4.606 \leqslant t \leqslant 7.355$, $p < 0.05$; CO_2 production by methanotrophs: 4 °C: $4.598 \leqslant t \leqslant 5.955$, $p < 0.05$, 12 °C: $4.341 \leqslant t \leqslant 12.416$, $p < 0.05$; CO_2 production by basal respiration: 4 °C: $t = 5.126$, $p < 0.05$).

The fact that the MOPs in Ämmässuo were on average 10-fold higher (4.16 μmol g_{dw}^{-1} h^{-1}) than the MOPs in Tarastenjärvi (0.41 μmol g_{dw}^{-1} h^{-1}, excluding Tj4), could be resulting from the Ämmässuo cover material, compost. Compost is considered as suitable support material for methanotrophs due to its beneficial characteristics (Huber-Humer et al., 2011) and, accordingly, the Ämmässuo compost samples studied here had higher moisture and organic matter contents on average than the samples of other soil types. Furthermore, the compost samples collected from Ämmässuo contained on average more NO_3^- than the soil samples of Tarastenjärvi, which was shown to have a significant positive linear relationship with MOP by the MLR analysis. The MOPs recorded for compost in Ämmässuo (1.58–5.98 μmol g_{dw}^{-1} h^{-1}) agreed well with the MOPs reported for composted materials in the literature: 6.5 μmol g_{dw}^{-1} h^{-1} for garden waste composted for 6 months (Mor et al., 2006) and 6.8 μmol g_{dw}^{-1} h^{-1} for a mixture of garden and kitchen waste composted for 6–8 months (Scheutz et al., 2014). The compost material in Ämmässuo had been

distributed to the landfill 2–5 years prior to sampling. This proves that ageing compost, such as compost in Ämmässuo, is able to support MOPs comparable to those of freshly composted samples (Mor et al., 2006; Scheutz et al., 2014) for several years. The MOPs measured here were higher than has been previously reported for compost after operation of 5 years as a biocover (2.5 μmol g_{dw}^{-1} h^{-1}) (Einola et al., 2007).

Even though the soil and sand samples collected from Tarastenjärvi contained less nutrients and organic matter than the samples from Ämmässuo, they exhibited MOPs ranging from 0.08 to 0.59 μmol CH_4 g_{dw}^{-1} h^{-1} (excluding Tj4). The MOPs recorded in this study compared well with the MOPs reported for soil materials in the literature, which have generally been lower than the MOPs reported for compost: 0.17 μmol g_{dw}^{-1} h^{-1} for sandy clay (Whalen et al., 1990), 0.0073–0.57 μmol g_{dw}^{-1} h^{-1} for sandy loam (Börjesson et al., 1998; Spokas and Bogner, 2011) and 1.00 μmol g_{dw}^{-1} h^{-1} for landfill soil with coarse sand (Kightley et al., 1995). The reason for MOPs often being greater in compost than in soil, is the higher
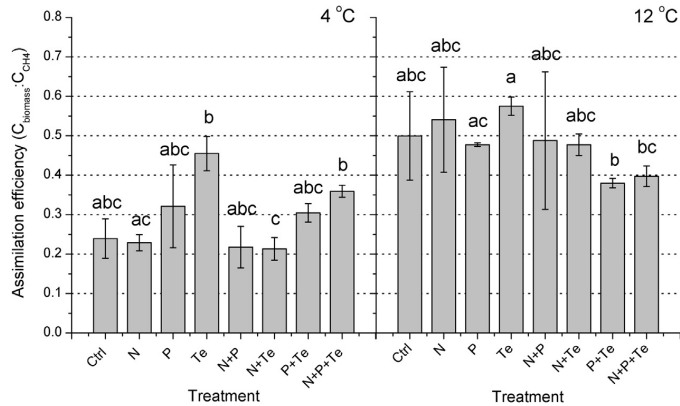
Fig. 3. Carbon assimilation efficiencies of methanotrophs in the untreated control sample (Ctrl) and in samples treated with nutrient solutions (N = nitrogen, P = phosphorus, Te = trace elements) incubated at 4 °C and 12 °C (mean values ± SE, $n = 2$). Different small alphabets indicate statistically significant difference between mean values (Student's 2-tailed t-test, df = 2, 4 °C: $4.422 \leqslant t \leqslant 5.060$, $p < 0.05$; 12 °C: $5.105 \leqslant t \leqslant 7.512$, $p < 0.05$).

nutrient content, specific surface area and air-filled porosity of compost (Huber-Humer et al., 2009). In addition, the finer texture of the soil material causes compaction of the structure and, consequently, retardation of the gas transfer between soil and atmosphere (Rose et al., 2012; Tanthachoon et al., 2008). The reduction in the size of soil particles in the course of time is caused by biological degradation processes (Scheutz et al., 2009). The organic matter and nutrient contents and, consequently, the BR were on average lower in Tarastenjärvi than in Ämmässuo indicating that the soil in Tarastenjärvi possessed conditions less favourable for methanotrophs.

The present results suggest that besides soil type and the analysed soil characteristics, there are also other factors which were not included in this study (such as ambient CH_4 loading), but which seem to have a significant effect on the MOP. The sample comprising of 20-years-old soil from Tarastenjärvi landfill had a higher MOP than any of the compost samples in Ämmässuo in spite of the low nutrient content of the soil (Tj4; 7.21 µmol CH_4 g_{dw}^{-1} h^{-1}). In addition, the MOP of a sample collected from a 1-year-old compost layer in Tarastenjärvi (Tj6; 0.91 µmol CH_4 g_{dw}^{-1} h^{-1}) was lower than in other compost samples (As1–As3) even though the organic matter content and BR rate of this sample were of the same order of magnitude. As the measured characteristics do not explain these MOPs, one explanation could be that the ambient concentration of CH_4 varied in these sampling locations. The soil sample with exceptionally high MOP (Tj4) was collected from landfill cover reported to have high CH_4 emission, whereas the compost cover (Tj6) was not found to emit CH_4 (Tampere Regional Solid Waste Management Ltd. emission follow-up survey; data not shown). The well-established population of methanotrophs in a permanent soil cover of a landfill have been shown to be able to respond to the increased substrate availability by increasing their CH_4 oxidation rate to manifold (Bogner et al., 1997).

To our knowledge, the present study is the first one showing evidence that the correlation between MOPs and BR rates is statistically significant, and that the compost and soil in landfill cover exhibiting high activity of heterotrophs can support high methanotrophic activity (e.g. As2 10–20, Tj4). The fact that the MOPs correlate positively with the BR rates (the more active the heterotrophs were, the higher the MOP measured) is contradictory to earlier findings (Huber-Humer et al., 2011; Mor et al., 2006). High BR activity is often considered undesirable as the competition over O_2 between methanotrophs and heterotrophs might limit the activity of methanotrophs (Huber-Humer et al.,

2011, 2009). One explanation for the correlation could be that heterotrophs transform the organic matter-bound nitrogen and phosphorus to its inorganic forms by degrading organic compounds, which can then be utilised by plants and bacteria including methanotrophs (Ehrlich, 1996; Prescott et al., 2005). Thus, the methanotrophs would be benefitting directly from the activity of heterotrophs. On the other hand, as discussed earlier, BR acts as an indicator of the stage of mineralisation and physical structure of the soil material (Mor et al., 2006; Rose et al., 2012). Therefore, it could be used as an indirect indicator of the suitability of the soil material for methanotrophic and other bacterial activity. However, the observed correlation of MOP and BR does not reveal the causality of these factors and, thus, more research is needed to show the validity of the discussed interactions.

The nutrient manipulation assay showed that, despite the NO_3^- content of the soil had the second strongest unique contribution to the MOP of the 12 studied cover materials (MLR analysis), the addition of N solution reduced the MOPs of the samples in nutrient manipulation assays. The concentration of NO_3^- added to the samples here (600–700 mg kg_{dw}^{-1}) was lower than the reported inhibitory value for landfill biocover soil (1200 mg kg_{dw}^{-1}) (Wang et al., 2011), so it was not expected to cause inhibition. A more likely explanation for the observed inhibition is too high ionic concentration of the soil water in the studied samples. The N solution contained also other compounds in addition to KNO_3 ($MgSO_4$, $CaCl_2$) and, therefore, the total amount of ions added to the samples in the N solution (1400 mg kg_{dw}^{-1} at 4 °C, 1600 mg kg_{dw}^{-1} at 12 °C) was higher than in the P or Te solutions (P: $\leqslant 1100$ mg kg_{dw}^{-1} at 4 °C, $\leqslant 1300$ mg kg_{dw}^{-1} at 12 °C). Methanotrophs are able to tolerate high ionic concentration in the soil and, when measured as conductivity, the recommended maximum total ion content of compost material is 4 mS cm^{-1} (Huber-Humer et al., 2009). The electrical conductivity of the samples was not measured after the experiment, but it was originally high in the sample, 2.0 µS cm^{-1} (As2 40–50), following from the high total anionic content, ~8500 mg kg_{dw}^{-1}. Therefore, it could have become inhibitive high for methanotrophs after addition of N solution (on average 10,000 mg kg_{dw}^{-1}). The inhibiting effect of high ionic concentration has been reported overcoming the stimulative effect of NO_3^- earlier in humisol samples amended with $NaNO_3$ and corresponding amount of NaCl – first the addition of $NaNO_3$ stimulated methanotrophs, but then the inhibition occurred at the same concentration of both compounds (Dunfield and Knowles, 1995).

The results of the nutrient manipulation assay indicate that MOPs and growth efficiencies of methanotrophs could be enhanced by ~16% at low temperatures (4 °C) by adding Te into the soil. The Te solution used in the assay contained several micro elements important for methanotrophs growth and expression and activity of MMO enzymes (Cu, Fe, Zn, Mn, Ni, Mo) (Nikiema et al., 2013). In the presence of Cu, the particulate form of MMO (pMMO) representative for type I methanotrophs is synthetized, whereas in the absence of Cu, Fe is utilised for synthesis of the soluble form of MMO (sMMO) representative for type II methanotrophs (Glass and Orphan, 2012). Here, the Te addition increased the MOP and carbon assimilation efficiency of methanotrophs at 4 °C probably due to activation of psychrotolerant type I methanotrophs that benefit from the added Cu. On the contrary, the Te addition did not increase the MOPs in samples incubated at 12 °C suggesting that the Fe content of the soil was originally sufficient enough for enabling the activity of type II methanotrophs. The assumption that only the type I methanotrophs were active at 4 °C would explain why the N additions did not inhibit the MOP to such great extent in samples incubated at 4 °C than in the samples incubated at 12 °C; type I methanotrophs are reported to have better tolerance towards high salt concentrations than type II methanotrophs (Reay et al., 2005; van der Ha et al., 2010). It has been earlier demonstrated that Cu addition has an enhancing effect on MOP and biomass production of methanotrophs in batch assays with laboratory cultures (Van der Ha et al., 2013; 2010), rice paddy soil (Mohanty et al., 2000) and also landfill soil (Lee et al., 2009), but all those assays have been conducted in 20–30 °C. To our knowledge, the present study is the first one to bring into question the role of Cu in stimulating the pMMO catalysed metabolic route of methanotrophs in landfill soil at low temperature.

The addition of PO_4^{3-} did not have a clearly distinguishable effect on the MOPs of the samples during the nutrient manipulation assay at either temperature (4, 12 °C). The original concentration of PO_4^{3-} in the soil used for nutrient manipulation assay was considerably less (As2 40–50; <2 mg kg_{dw}^{-1}) than what is recommended for methanotrophs in landfill cover material, $\geqslant$55 mg PO_4^{3-} kg^{-1} (calculated from $\geqslant$3000 mg P_{total} kg^{-1} (Huber-Humer et al., 2011) by assuming that 0.6% of P_{total} is biologically available (Nikiema et al., 2013) and mostly PO_4^{3-} (Ehrlich, 1996)). Thus, it was assumed that by adding 870–1040 mg PO_4^{3-} kg_{dw}^{-1} to the soil the MOP would be enhanced, but the addition did not induce a clear stimulation or inhibition of the methanotrophs. Similarly, no effect on MOP was reported after addition of 300 mg PO_4^{3-} kg_{ww}^{-1} to coarse sand samples (Kightley et al., 1995). On the contrary, PO_4^{3-} has also been found to stimulate the methanotrophic activity in tropical forest soil during field experiment (460 kg PO_4^{3-} ha^{-1} yr^{-1}) (Zhang et al., 2011) and in landfill cover soil when added as part of NPK fertilizer in laboratory batch assays (18–60 mg P kg_{dw}^{-1}) (Jugnia et al., 2012). Comparison of the results of this study between the earlier published results is difficult due to the variation in the used units and scarcity of the available studies. Thus, the effect mechanism of PO_4^{3-} addition on methanotrophs should be studied more in detail. It seems, however, that the methanotrophs' demand for PO_4^{3-} is not considerably high as the soil and compost samples of Ämmässuo and Tarastenjärvi were able to exhibit notably high MOPs (e.g. As1 10–20; 5.68 µmol g_{dw}^{-1} h^{-1}) in spite of being depleted from PO_4^{3-}.

5. Conclusions

The purpose of this study was to screen the MOPs of different materials used in landfill cover structures and to determine and manipulate the factors affecting the MOPs in order to obtain information that can be utilised in emission control. The compost in the

biocovers designed for mitigating CH_4 emissions was shown to be the most efficient in oxidising CH_4 (1.58–5.98 µmol g_{dw}^{-1} h^{-1}). The materials from the other cover structures not merely designed for supporting CH_4 oxidation (final cover soil, gas well foundation sand) were also capable of oxidising CH_4, but with rates 10-fold lower. The results suggested, however, that the MOP of final cover soil could become increased to manifold as a response to a high point loading of CH_4. The material failing to support heterotrophic activity due to low organic matter content or fine texture is probably not suitable material for supporting CH_4 oxidation as the methanotrophic activity was found to correlate significantly with heterotrophic activity. Furthermore, it was shown that methanotrophs are more dependent on the availability of NO_3^- than the availability of PO_4^{3-} in the cover materials and, thus, this should be considered in the material selection for new landfill covers. Also, it seems that favouring materials with high content of trace elements would be beneficial for improving the emission control at cold temperature (4 °C).

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Evaluation of methods for enhancing methane oxidation via increased soil air capacity and nutrient content in simulated landfill soil cover

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Evaluation of methods for enhancing methane oxidation via increased soil air capacity and nutrient content in simulated landfill soil cover

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ABSTRACT

Landfill soil covers and methanotrophs therein have potential to act as final sinks of the greenhouse gas methane (CH_4) generated in landfills, but soil characteristics in landfills might not support methanotrophic activity due to poor soil material selection or mineralisation over time. Hence, our aim was to determine the performance of mineral landfill soil under simulated CH_4 flux and screen methods for elevating the CH_4 elimination capacity (EC) of soil. The methods tested during the column experiment were inorganic fertilisation (nitrate, phosphate, sulphate, copper), decompaction and amelioration of the soil with compost. The addition of compost proved to be the most effective method for increasing the CH_4 EC of soil, increasing from 55 to 189 $g\,m^{-2}\,d^{-1}$ relative to the untreated control soil. This increase could be attributed to increased air capacity, concentration of soil nutrients and number of cultivable methanotrophs. Also, soil water-holding capacity was identified as a more crucial factor for methanotrophic activity than total porosity. Inorganic fertilisation and decompaction induced only a temporary increase in CH_4 EC, likely resulting from the temporary supply of fertiliser to the nutrient-deprived soil. In conclusion, we suggest that compost amelioration (22 w-%) could be useful for restoring CH_4 EC of old landfill covers as an aftercare action to control environmental impacts of closed landfills.

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1. Introduction

Methane (CH_4) emitted from landfills represents the second largest contributor to total CH_4 emissions in European Union countries (94 out of 468 Tg CO_2-equivalents in 2013) (EEA, 2015). To prevent emission of CH_4 into the atmosphere, landfill gas is often recovered via gas extraction systems or in some cases using engineered cover structures that promote biological CH_4 oxidation (Sadasivam and Reddy, 2014). Oxidation by methanotrophs leads to transformation of 10–100% of CH_4 produced in landfills into carbon dioxide (CO_2), water and biomass; the fraction that is not oxidised is released to the atmosphere (Humer and Lechner, 1999).

The activity of methanotrophs, and consequently the CH_4 elimination capacity (CH_4 EC) of landfill cover soil, is affected by various factors among which the supply of the main substrates, CH_4 and oxygen (O_2), is the most crucial. Diffusion of gases in the landfill cover is passive and is more rapid in materials with high air capacity, that is, in materials with low bulk density, high water-holding

capacity (WHC) and large proportion of air-filled pore space (AFPS) (Bohn et al., 2011; Smith et al., 2000). The CH_4 EC of such materials is often high (Huber-Humer et al., 2011). As for the other growth factors, methanotrophs require water in their surroundings to maintain their metabolic activities; the optimal moisture content is 50–100% of WHC of the material (Huber-Humer et al., 2009; Mancebo and Hettiaratchi, 2015). Methanotrophs also require nitrogen and phosphorus for synthesis of different cell constitutes (Humer and Lechner, 1999) and micronutrients such as copper and iron for synthesis of enzymes (Glass and Orphan, 2012).

Landfill covers can be designed to support CH_4 oxidation by using materials such as compost that meet most of the requirements of methanotrophs and that possess a high specific surface area for microbial attachment (Huber-Humer et al., 2009). However, material selection is often based on the cost and availability of material, and different mineral soils and spoils are commonly used to construct landfill covers (Sadasivam and Reddy, 2014). Characteristics of these materials are often not optimal for methanotrophs, leading to low CH_4 EC. For instance, fine particle sizes result in higher bulk density and lower AFPS, lack of organic matter decreases the WHC (Olorunfemi et al., 2016) and nutrient content is rather poor (Maanoja and Rintala, 2015). Soil structure

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and its physical, chemical and physicochemical properties also transforms naturally via biological mineralisation of organic matter over time. This leads to the possibility that CH_4 EC of ageing landfill covers will decrease with time, resulting in the release of CH_4 from the landfill to the atmosphere.

In this work, our aim was to study the CH_4 EC of landfill cover consisting of mineral soil (organic carbon <20 w-%; Toth et al., 2008) and screen methods for improving its CH_4 EC. We focused on increasing the air capacity and nutrient content of the mineral soil, focusing to this end on three methods: (1) fertilisation with inorganic nutrients, (2) mechanical decompaction of the soil structure and (3) amelioration of the soil with compost, which acts as a source of organic nutrients and as a bulking agent. The rationale for this study was to produce information on technologies that can be used to prevent the harmful influence that landfills can have on the environment without appropriate aftercare.

2. Materials and methods

2.1. Landfill cover soil

Cover soil was obtained from a 20-year-old final cover at Tarastenjärvi landfill (Tampere, Finland). This soil was selected from among six previously screened cover soil samples (Maanoja and Rintala, 2015) because it exhibited the highest CH_4 oxidation potential (MOP). The granulometric composition of the soil was 51.3% gravel, 40.4% sand and 8.3% silt. Soil was sieved into size fractions <19 mm, corresponding to 81% of the total weight of the soil; the separated fraction, consisting of stones and wood debris, was removed to prevent paths for preferential gas flow in the columns. Sieving altered the granulometric composition of the soil to 39.8% gravel, 49.9% sand and 10.3% silt, leading to classification as well-graded silty gravelly sand (Whitlow, 2001). Distilled H_2O was added to the sieved soil to increase the moisture content from 23 to 34% of wet weight (ww) corresponding to 50% of the WHC of the soil (79% dry weight (dw)). The soil contained 10.4% dw organic matter and had a pH of 5.84.

2.2. Column setup for simulating landfill cover

Soil was placed in two acrylic columns (height of 60 cm, inner diameter of 14.2 cm; Fig. 1), which served as treatment and control columns, respectively. Replications were not used due to the large quantity of soil in each column, which was considered representative based on the designs of similar studies (De Visscher et al., 1999; Park et al., 2002; Rose et al., 2012; Wilshusen et al., 2004). Columns were equipped with gas sampling ports at the gas inlet and outlet and on the side of each column. The columns were filled with 9.33 kg wet soil to a total bed height of 45 cm, corresponding to wet bulk density of 1.31 kg L^{-1} (Table 1); this value was selected according to the literature values (1.24–1.35 kg L^{-1}; Huber-Humer et al., 2011).

To simulate the operation of a landfill cover, synthetic landfill gas (CH_4:CO_2 50:50 v-%, Aga Ltd.) was introduced to the bottom of the column through a gravel layer. Pressurised air was introduced to the empty space above the soil bed and diffused passively into the soil. Initially (days 0–24), the flow rate of landfill gas was 3.8 mL min^{-1} corresponding to an area-based loading rate of 110 g CH_4 m^{-2} d^{-1} (Table 1). On day 25, the loading rate was increased in both columns to 210 g CH_4 m^{-2} d^{-1} (7.3 mL min^{-1}) and maintained for the rest of the experiment; this exceeded the CH_4 EC of the columns and allowed for testing the soil improvement methods. The increased flow rate replaced the gas inside the AFPS of the soil 7.37 times daily instead of 3.84 times daily. The CH_4 loading rates
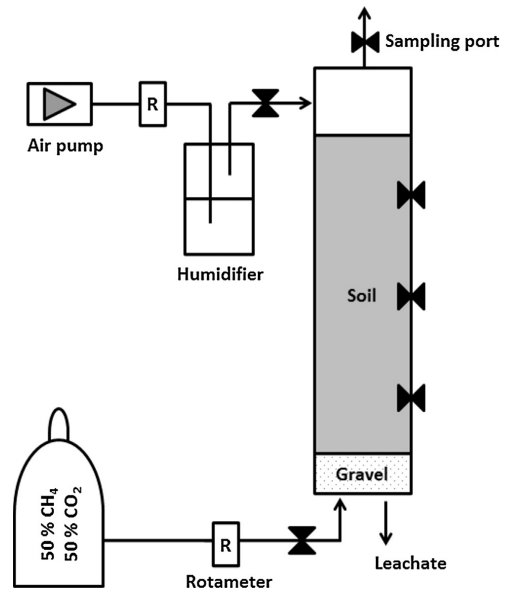
Fig. 1. Laboratory-scale column setup used for simulating performance of a landfill cover.

were chosen according to the rates reported for different municipal solid waste landfills, which range from 21.5 g CH_4 m^{-2} d^{-1} (Einola et al., 2008) to 518 g CH_4 m^{-2} d^{-1} (Reddy et al., 2014). The airflow rate for O_2 loading was adjusted to correspond to a molar ratio of 4:1 O_2:CH_4 to support complete oxidation of CH_4 (Einola et al., 2008).

2.3. Methods for improving CH_4 elimination

Three methods of improving CH_4 elimination from the soil were tested with the treatment column on four occasions: fertilisation (days 64–79 and 113–114), decompaction of the soil bed by mixing (day 97) and amelioration of soil with compost (day 120). As for the control column, distilled water was added instead of fertilisers (days 64–79 and 113–114) and the soil bed was decompacted without addition of compost (days 97 and 120) (Table 1). Soil samples (300 g at day 97 and 100 g at day 120) were collected from the columns prior to applying each method for determination of methanotrophic activity and the chemical characteristics of the soil.

Fertilisation was carried out twice, at days 64–79 and 113–114, by injecting nutrient solutions into the column from the top and through the side ports (Table 1). In the first fertilisation (days 64–79), nutrients added to the treatment column included 150 mg PO_4^{3-}, 400 mg NO_3^-, 500 mg SO_4^{2-} and 0.385 mg Cu^{2+} per kilogram of soil (0.64 mg Cu^{2+} L^{-1} of soil water). These additions increased the initial phosphate and nitrate contents of the soil ten- and 20-fold, respectively; concentrations of copper and sulphate were adjusted to optimal for methanotrophs (Huber-Humer et al., 2009; van der Ha et al., 2010). The first fertilisation did not induce an increase in CH_4 EC; therefore, in the second fertilisation (days 113–114), the amount of nutrients was increased to 400 mg PO_4^{3-}, 600 mg NO_3^- and 750 mg SO_4^{2-} per kilogram of soil. The concentration of copper was not increased (0.370 mg kg^{-1})

Table 1

Operational data for the laboratory columns (control and treatment) used to study the effect of soil improvement methods on CH_4 elimination capacity of landfill cover soil.

Characteristics of the soil in the columns					
Time	(day)	0–97	97–120	120–148	120–148
Column		Both	Both	Control	Treatment
Volume of the soil inside the column	(L)	7.13	6.81	6.97	7.21
Wet weight	(kg)	9.3	8.9	8.8	7.9
Dry weight	(kg)	6.2	5.8	5.6	4.6
Wet bulk density of the soil	(kg L^{-1})	1.31	1.31	1.26	1.10
Dry bulk density of the soil	(kg L^{-1})	0.87	0.86	0.83	0.64
Total porosity (φ)	(kg L^{-1})	0.64	0.64	0.65	0.70
Air-filled pore space (φ_g)	(kg L^{-1})	0.20	0.20	0.23	0.24
Operation of the columns					
Time	(day)	0–24	25–148		
Column		Both	Both		
Flow rate, LFG (50:50 CH$_4$:CO$_2$ v-%)	(mL min^{-1})	3.8	7.3		
Flow rate, air (21 v-% O$_2$)	(mL min^{-1})	33.2	68.6		
Area-based CH$_4$ load	(g m^{-2} d^{-1})	110	210		
Area-based O$_2$ load	(g m^{-2} d^{-1})	798	1600		
Methods applied for improving the soil					
Time	(day)	64–79	97	113–114	120
Control column		Irrigation with H$_2$O	Decompaction	Irrigation with H$_2$O	Decompaction
Treatment column		Fertilisation with inorganic nutrients	Decompaction	Fertilisation with inorganic nutrients	Amelioration with compost
Theoretical concentration of nutrients in the treatment column after application of the improvement method[a]	(mg PO$_4^{3-}$ kg^{-1})	164	–	425	49
	(mg NO$_3^-$ kg^{-1})	402	–	637	1311
	(mg Cu^{2+} kg^{-1})	0.547	–	0.528	0.255
	(mg SO$_4^{2-}$ kg^{-1})	461	–	1180	1463

LFG, synthetic landfill gas; v-%, percentage by volume.

[a] Calculated concentration of nutrients after fertilisation or compost amelioration; the concentrations measured at the previous time point were summed with the amount of nutrients retained in the column after leaching (0.4–10.7% after the first fertilisation and 1.7–7.0% after the second fertilisation, data not shown).

to preventing inhibiting the expression of methane mono-oxygenase enzymes of type II methanotrophs (van der Ha et al., 2010). In the second fertilisation, the nutrients were added one day and the solution that leached from the column overnight was re-inserted in the column the next day to improve retention of the nutrients (Table 1).

Decompaction of the soil was carried out after 97 days. Soil was removed from the columns in three layers based on zones coloured by the bacterial growth. The orange-red zone was assumed to represent the active methanotrophic zone and the extracellular polysaccharides (EPS) excreted by methanotrophs (Humer and Lechner, 1999). The middle layer (depth of 10–27 cm) was coloured orange, while the top (0–10 cm) and bottom (27–45 cm) layers did not show distinct colour formations. The mass distribution of the layers was 1.36, 2.32 and 2.45 kg dw from top to bottom. The layers were mixed after collecting samples for determination of the MOP of the soil to homogenise the texture and the chemical and biological properties of the soil, after which it was re-packed into the columns to the initial bed height of 45 cm.

The soil of the treatment column was ameliorated with compost after 120 days in order to improve its air capacity and increase the content of nutrients and organic matter. The compost was obtained from Koukkujärvi landfill (Nokia, Finland) and consisted of woodchips and sewage sludge with a particle size of <20 mm; it contained 41.9% dw organic matter and had pH of 6.87. Soil was removed from the column and mixed with compost in a wet weight ratio of 70:30, corresponding to a dry weight ratio of 78:22. This ratio was selected to correspond a height ratio of 20:80 compost to soil layer in the column, which is representative of a potential amendment on a real landfill. The mixture was re-packed into the column to the initial bed height of 45 cm. Compost had lower dry bulk density than soil (0.30 vs. 0.87 kg L^{-1}), resulting in lower dry bulk density of the overall mixture (0.64 kg L^{-1}; Table 1).

2.4. Determination of activity and number of cultivable methanotrophs

Methanotroph activity was assessed by measuring the MOP of the soil collected from the column at depths of 0–10, 10–27 and 27–45 cm. The analysis was conducted in batch bottles (118 mL, n = 2) in which 6 g of moist soil was incubated under air with 7% CH$_4$ (v/v) at 22 ± 2 °C. In addition, two soil samples were incubated without added CH$_4$ in order to determine the production of CO$_2$ by basal respiration of the decomposing heterotrophic soil bacteria. Incubation of the samples was ended when the concentration of CH$_4$ fell below the limit of detection (<0.1% v/v) of the gas chromatograph (GC). The incubation period ranged from 10 to 48 h depending on the activity of the methanotrophs in each sample.

The number of cultivable methanotrophs in the samples of soil collected after mixing the soil layers from different column depths was determined via the most probable number (MPN) method (Alexander, 1965). One sample of 11 g moist soil was mixed with 95 mL of nitrogen mineral salts (NMS) solution containing the following per litre of water: 1.0 g KNO$_3$, 1.1 g MgSO$_4$·7H$_2$O, 0.2 g CaCl$_2$, 0.3 g KH$_2$PO$_4$, 0.4 g Na$_2$HPO$_4$·2H$_2$O, 3.8 mg Fe-EDTA, 0.28 mg Na$_2$MoO$_4$·2H$_2$O, 0.20 mg CuSO$_4$·5H$_2$O, 0.50 mg FeSO$_4$·7H$_2$O, 0.40 mg ZnSO$_4$·7H$_2$O, 0.02 mg H$_3$BO$_3$, 0.05 mg CoCl$_2$·6H$_2$O, 0.28 mg EDTA-Na$_2$·2H$_2$O, 0.02 mg MnCl$_2$·4H$_2$O and 0.01 mg NiCl$_2$·6H$_2$O (pH 6.8). A series of ten-fold dilutions was prepared with NMS solution and one millilitre of each dilution was added to three parallel tubes together with 5 mL of NMS solution. Tubes were incubated with 10% CH$_4$ (v/v) under air for two weeks at 25 °C. The presence of methanotrophs was identified as depletion of CH$_4$ from the tube.

2.5. Analytical methods and calculations

The dry weight and moisture content of the soil samples were determined gravimetrically after drying the samples (5–7 g ww,

$n = 2$) overnight at 105 °C (SFS-EN 14346, 2007). The organic matter content was determined gravimetrically from the dried soil samples as loss of volatile solids on ignition after ignition at 550 °C for one hour (SFS-EN 15169, 2007). The particle size distribution of original landfill soil was determined by sieving (sieve openings 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0.063 mm) a dried soil sample (2.4 kg) after separating the fine fraction (<0.063 mm) by rinsing with water (SFS-EN 933-1, 2012). The particle size distribution of the fine fraction was determined via a sedigraph (Sedi-Graph III Plus Micromeritics). WHC was determined in moist soil compacted into plastic cylinders (118 mL, $n = 1$–2) by measuring the constant weight after soaking for 24 h and draining for two hours (FCQAO, 2003). The pH of the soil was determined from slurries of soil and 0.01 M CaCl₂ (1:2.5 v/v, $n = 2$–3) (SFS-ISO 10390, 2007).

Concentration of CH_4, CO_2 and N_2 in the soil air and incubation bottles was determined by analysing samples with a GC coupled to a thermal conductivity detector (GC-TCD) (Maanoja and Rintala, 2015). Concentration of O_2 was analysed via a GC-TCD with a Supelco Carboxen-1000 60/80 column and with the injector and detector set at 225 and 230 °C, respectively. The oven ramped from 45 to 225 °C at 180 °C min⁻¹ after holding at 45 °C for 4.3 min. The carrier gas flow was 30 mL He min⁻¹.

The MOP and CO_2 production rate of the methanotrophic and heterotrophic bacteria in the batch experiment were determined as the slope of the linear part of the regression curve with concentration as a function of time. The production of CO_2 by methanotrophs was calculated from the difference of the CO_2 production rates measured from the MOP and basal respiration samples, as described previously (Maanoja and Rintala, 2015). The molar ratio of produced CO_2 per oxidised CH_4 was calculated for the batch bottles and for the columns as a measure of the activity of methanotrophic and heterotrophic bacteria; the theoretical maximum molar yield of CO_2 per CH_4 in methane oxidation is 1; when the value is greater than this, other bacteria are contributing to CO_2 production (Einola et al., 2008).

The CH_4 EC (g m⁻² d⁻¹) of the soil columns was calculated according to Eq. (1), where q is the flux (L min⁻¹), M is the molar mass, 1440 is a conversion factor from minutes to days, V_m is the molar volume calculated separately for each measuring point with daily atmospheric pressure and temperature (L mol⁻¹) and A_{col} is the area of the column (0.0158 m²). Production of CO_2 was calculated in a similar manner. CH_4 RE (%) was calculated for the soil columns according to Eq. (2).

$$CH_4 \ elimination \ capacity = \frac{(q_{outlet} - q_{inlet}) \cdot M_{CH4} \cdot 1440}{-1 \cdot V_m \cdot A_{col}} \quad (1)$$

$$CH_4 \ removal \ efficiency = \frac{q_{inlet} - q_{outlet}}{q_{inlet}} \cdot 100 \quad (2)$$

To determine the soluble nutrient content (nitrate, phosphate, sulphate) of the samples, a filtered water extract of the air dried soil (soil:water 1:4 or 1:1, w/v, $n = 2$) was analysed via an ion chromatograph (SFS-EN ISO 10304, 2009). The copper content in the soil was determined from a filtered (0.45 μm) supernatant of the air-dried soil and 0.01 M CaCl₂ slurry (1:10 w/v, $n = 2$) (Quevauviller, 1998) with an atomic absorption spectrophotometer at 324.75 nm.

The total porosity (φ) and AFPS (φ_g) were calculated for the soil material in the columns according to Eqs. (3) and (4), where ρ is wet bulk density, w_s is the weight fraction of wet solids, ρ_s is particle density (2413 kg m⁻³ for soil, 1554 kg m⁻³ for compost) and ρ_w is water density (Mancebo and Hettiaratchi, 2015):

$$\varphi = 1 - \frac{\rho \cdot w_s}{\rho_s} \quad (3)$$

$$\varphi_g = \varphi - \frac{\rho(1 - w_s)}{\rho_w} \quad (4)$$

3. Results

3.1. CH_4 elimination and production of CO_2 in the columns

During the first week of operation (days 0–7), the added CH_4 (110 g m⁻² d⁻¹) was eliminated at a rate of 94.8–97.2 g m⁻² d⁻¹ (RE 86–89%) in the two simultaneously operated columns (Fig. 2A, B). After the first week, the CH_4 EC decreased and stabilised in both columns at 54.4–55.3 g m⁻² d⁻¹ (days 8–64), remaining constant even after the CH_4 loading was increased to 210 g m⁻² d⁻¹ at day 24 (RE decreased from 55 to 27%).

Fertilisation of the treatment column and simultaneous irrigation of the control column between days 64 and 79 did not notably affect CH_4 EC. In the control column, the CH_4 EC remained at 50.4 g m⁻² d⁻¹ on average (RE 24%) during and after irrigation (days 64–97). In the treatment column, CH_4 EC fluctuated between 36.1 and 98.3 g m⁻² d⁻¹ (RE 17–45%) during the first fertilisation (days 64–79) and peaked at a maximum of 138.4 g m⁻² d⁻¹ (RE 65%) at day 90 before decreasing back to 43.9 g m⁻² d⁻¹ (RE 21%) by day 97.

The soil bed was removed from both columns and decompacted using the same method at day 97; by day 108, the CH_4 EC in the treatment column increased from 43.9 to 203 g m⁻² d⁻¹ (RE 100%). Shortly thereafter, it started decreasing and stabilised at 105.0 g m⁻² d⁻¹ by day 112 (RE 49%). Decompaction did not affect the CH_4 EC of the control column.

The second fertilisation of the treatment column and irrigation of the control column at days 113–114 induced a small increase in the CH_4 EC of the treatment column from 105.0 to 133.8 g m⁻² d⁻¹ (RE from 49 to 61%, days 114–120). In the control column, the CH_4 EC decreased from 53.3 to 34.7 g m⁻² d⁻¹ (RE from 25 to 16%, days 114–120).

Following amelioration of the soil in the treatment column with compost at day 120, the CH_4 EC increased to between 165.3 and 207.9 g m⁻² d⁻¹ and averaged at 189.1 g m⁻² d⁻¹ (RE 88%) for the remaining time of the experiment (days 122–148). Due to the simultaneous decompaction of the soil bed in the control column (without the addition of compost), CH_4 EC temporarily increased and stabilised back at the same level or 57.4 g m⁻² d⁻¹ (RE 27%, days 122–148) which had prevailed throughout the experiment.

In the treatment column, the production of CO_2 was 204 g m⁻² d⁻¹ on average before application of any the improvement methods (days 0–64); after the first fertilisation, CO_2 production fell to 110 g m⁻² d⁻¹ (days 64–90) (Fig. 2D). A few days before decompaction of the soil, the production of CO_2 peaked at 405 g m⁻² d⁻¹ (day 97) and remained unstable, ranging from 108 to 469 g m⁻² d⁻¹ until the end of the experiment (days 97–148). In the control column, the production of CO_2 remained rather stable throughout the experiment (Fig. 2C). Before application of the improvement methods, the production of CO_2 averaged 208 g m⁻² d⁻¹ (days 0–64), which decreased to 117 g m⁻² d⁻¹ after the first irrigation (days 64–90) and then increased slowly back to 200 g m⁻² d⁻¹ by the end of the experiment (days 97–148).

In the treatment column, the molar ratio of CO_2 produced to CH_4 oxidised ranged from 0.27 to 4.15 (mean 1.69) before application of the improvement methods (days 0–64) (Fig. 2F). After the beginning of the first fertilisation, the ratio stabilised at 0.63 on average, where it remained until the end of the experiment (days 65–148) with the exception of two occasions: it peaked to 3.37 and 3.42, respectively, on days 111 and 120. In the control column, the molar ratio of CO_2 to CH_4 ranged from 0.18 to 2.33 (mean 1.24) before application of the treatment methods (days 0–64) (Fig. 2E).
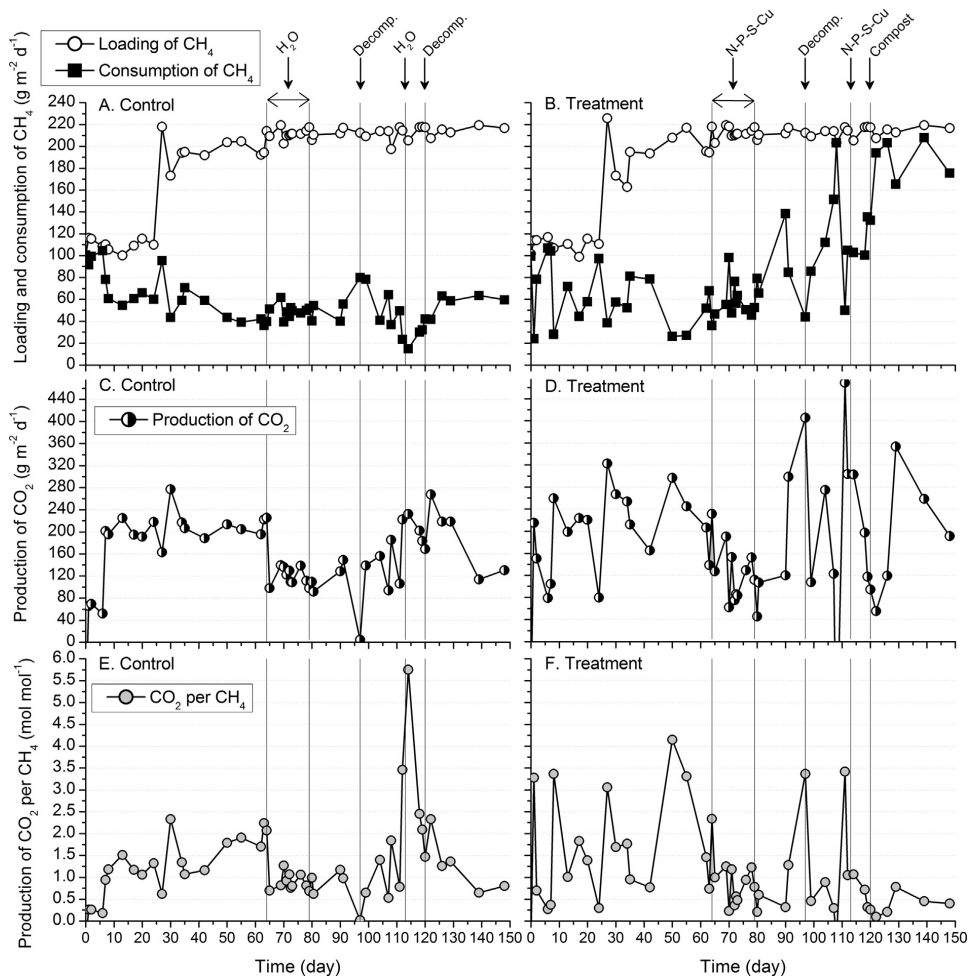
Fig. 2. (A, B) CH_4 loading and consumption rates, (C, D) CO_2 production rates and (E, F) molar ratios of CO_2 produced to CH_4 oxidised during the column experiment. Time points for the applied soil improvement methods are shown with vertical lines; methods applied to the control column were irrigation ('H_2O') and decompaction ('Decomp.'), and methods applied to the treatment column were fertilisation ('N-P-S-Cu'), decompaction ('Decomp.') and soil amelioration with compost ('Compost').

After the first irrigation, the ratio decreased to 0.90 on average (days 65–112) and then spiked to 5.75 after the second irrigation (at day 114). After this brief peak, the molar ratio decreased to an average of 1.31 (days 120–148).

3.2. Activity of methanotrophs and basal respiration

The initial MOP of the soil inserted in the columns was 25.7 µg CH_4 g^{-1} h^{-1} with production of CO_2 by basal respiration of 14.9 µg CO_2 g^{-1} h^{-1} (Table 2). After 97 days and application of either nutrients or water (days 64–79), the MOPs slightly increased in both columns, to 30.1 µg CH_4 g^{-1} h^{-1} (min 22.3, max 37.3 µg g^{-1} h^{-1}) on average in the treatment column and to 29.0 µg CH_4 g^{-1} h^{-1} (min 23.4, max 31.8 µg g^{-1} h^{-1}) on average in the control column. Similarly, the basal respiration rate increased to 36.5 and 31.5 µg CO_2 g^{-1} h^{-1} in the soil of the treatment and control columns,

respectively. The highest MOPs and basal respiration rates were measured in the top layer (0–10 cm) and the lowest rates were measured in the bottom layer (27–45 cm) of the soil bed.

At the end of the experiment, the amended soil in the treatment column exhibited considerably high methanotrophic activity (135.9 µg CH_4 g^{-1} h^{-1}) and moderate basal respiration rate (22.5 µg CO_2 g^{-1} h^{-1}). Consequently, the MOPs of the treatment column soil increased to 25.9–99.7 µg CH_4 g^{-1} h^{-1} and the basal respiration rate increased to 35.0–66.7 µg CO_2 g^{-1} h^{-1} (Table 2). The soil in the control column, which had been irrigated (days 113–114) and decompacted (day 120), showed no notable difference in MOP compared to that measured at day 97. Basal respiration decreased to 24.2 µg CO_2 g^{-1} h^{-1}.

The molar ratio of CO_2 produced to CH_4 oxidised was calculated for the batch samples. Initially, the ratio was higher in the landfill soil (0.68) than in the compost (0.53) (Table 2). In the treatment

Table 2
Number of methanotrophs, CH_4 oxidation potentials (MOP) and CO_2 production rates of methanotrophs and other soil bacteria measured from the landfill cover soil, compost and soil-compost mixture at different soil depths in the columns before the start of the experiment (day 0), after fertilisation or irrigation (day 97) and after compost addition or decompaction (day 148) (mean ± standard deviation, $n = 1$–6).

Parameter	Cover soil	Compost	Control[a]		Treatment[a]	
Day	0	0	97	148	97	148
MPN (10^6 cells g^{-1})	5.9	29.6	3.1	6.4	13.0	705
MOP ($\mu g\ CH_4\ g\ soil^{-1}\ h^{-1}$)						
0–10 cm	NA[b]	NA[b]	31.8 ± 1.04	33.6 ± 0.59	37.3 ± 4.68	99.7 ± 2.82
10–27 cm	NA[b]	NA[b]	31.9 ± 1.73	27.8 ± 0.77	30.7 ± 1.03	49.8 ± 1.10
27–45 cm	NA[b]	NA[b]	23.4 ± 0.26	19.5 ± 0.25	22.3 ± 0.53	25.9 ± 4.07
Mean	25.7 ± 2.97	135.9 ± 20.65	29.0 ± 4.46	27.0 ± 6.36	30.1 ± 7.08	58.5 ± 33.75
Production of CO_2 by basal respiration ($\mu g\ CO_2\ g\ soil^{-1}\ h^{-1}$)						
0–10 cm	NA[b]	NA[b]	42.2 ± 0.88	32.4 ± 0.24	60.1 ± 2.62	58.0 ± 6.58
10–27 cm	NA[b]	NA[b]	32.8 ± 0.32	24.9 ± 1.61	31.7 ± 1.01	66.7 ± 3.15
27–45 cm	NA[b]	NA[b]	19.5 ± 0.40	15.5 ± 2.62	17.6 ± 1.10	35.0 ± 0.05
Mean	14.9 ± 0.83	22.5 ± 1.38	31.5 ± 10.16	24.2 ± 7.70	36.5 ± 19.40	53.2 ± 14.99
Production of CO_2 in MOP samples: sum of methanotrophic and basal respiration ($\mu g\ CO_2\ g\ soil^{-1}\ h^{-1}$) and the contribution of methanotrophs [%]						
0–10 cm	NA[b]	NA[b]	79.1 ± 0.94 [46.6]	73.2 ± 1.76 [55.7]	113.0 ± 13.46 [46.8]	173.2 ± 0.95 [66.5]
10–27 cm	NA[b]	NA[b]	73.3 ± 3.48 [55.3]	59.3 ± 2.82 [58.0]	68.7 ± 3.77 [53.9]	121.6 ± 2.69 [45.1]
27–45 cm	NA[b]	NA[b]	50.3 ± 2.07 [61.2]	43.4 ± 1.02 [64.3]	48.8 ± 1.69 [63.9]	69.7 ± 5.42 [49.8]
Mean	50.0 ± 5.11 [70.2]	200.8 ± 31.47 [88.8]	67.5 ± 13.76 [53.3]	58.6 ± 13.52 [58.7]	76.8 ± 30.08 [52.5]	121.5 ± 46.43 [56.2]
Molar ratio of CO_2 produced to CH_4 oxidised in MOP samples (mol CO_2 mol CH_4^{-1})						
0–10 cm	NA[b]	NA[b]	1.06 ± 0.065	0.80 ± 0.046	1.31 ± 0.057	0.63 ± 0.019
10–27 cm	NA[b]	NA[b]	0.98 ± 0.018	0.78 ± 0.025	0.93 ± 0.037	0.89 ± 0.019
27–45 cm	NA[b]	NA[b]	0.78 ± 0.023	0.81 ± 0.008	0.80 ± 0.008	0.97 ± 0.055
Mean	0.68 ± 0.024	0.53 ± 0.0005	0.94 ± 0.135	0.79 ± 0.028	1.01 ± 0.240	0.83 ± 0.160

MPN, most probable number of methanotrophs; NA, not applicable.

[a] The control column was subjected to irrigation and decompaction, and the treatment column was subjected to fertilisation and compost addition before sampling at days 97 and 148, respectively.

[b] MOPs and production of CO_2 of the landfill cover soil and compost were analysed prior to adding them to the columns.

column, the molar ratio increased to 1.01 by day 97 after the first fertilisation (days 64–79) but decreased to 0.83 by day 148 after the second fertilisation and amelioration with compost (days 113–114 and 120). In the control column, the amount of CO_2 produced versus the amount of CH_4 oxidised showed a smaller increase than that of the treatment column by day 97, from 0.68 to 0.94, and decreased to 0.79 by day 148.

The number of cultivable methanotrophs was determined from the pooled and mixed soil layers. The soil initially contained 5.9×10^6 cells g^{-1}; and after 97 days, the MPN had increased in the soil of the treatment column (13.0×10^6 cells g^{-1}) and decreased in the soil of the control column (3.1×10^6 cells g^{-1}) (Table 2). The compost added to the treatment column at day 120 contained a high number of methanotrophs, 29.6×10^6 cells g^{-1}. The MPN of the treatment column increased to 705×10^6 cells g^{-1} by the end of the experiment (day 148), while the MPN of the control column remained at 6.4×10^6 cells g^{-1}.

3.3. Soil characteristics

Amelioration of soil with compost increased the amount of organic matter in the soil from 10.7 to 17.6% dw. In the control column, the organic matter content of the soil remained relatively unchanged throughout the experiment (9.1–10.1% dw) (Table 3). The moisture content of the soil remained close to the initial moisture content (33.6% ww) in both columns until amelioration of the soil with compost, which increased the moisture content of the treatment column to 40.8% ww and the WHC from 79 to 110% dw. Similarly, wet bulk density, total porosity and AFPS of both columns remained stable throughout the experiment (1.29 kg L^{-1}, 0.64 L L^{-1} and 0.21 L L^{-1} on average, respectively) until addition of compost to the treatment column decreased the wet bulk density to 1.10 kg L^{-1} and increased the total porosity and AFPS to 0.70 and 0.24 L L^{-1}, respectively (Table 1).

The gas concentration profiles of the columns were used to determine the diffusion and advection of the gases in the soil (Fig. 3). The best gas for estimating the penetration of gases to the soil was N_2 as it is not consumed or produced during CH_4 oxidation (Humer and Lechner, 1999). In both columns, the penetration of N_2 decreased slightly after increasing the CH_4 load at day 25 (Fig. 3A). In the control column, the penetration of N_2 did not changed despite application of the different soil improvement methods, and its concentration ranged from 14.3% at the deepest sampling depth (32.5 cm) to 46% at the shallowest sampling depth (12.5 cm) throughout the experiment. Similarly, in the treatment column, fertilisation and decompaction did not affect penetration of N_2, the concentration of which ranged from 13.5 to 38.9% in the soil. After the soil of the treatment column was amended with compost, the penetration of N_2 improved and the concentration increased at every depth (35.1–65.3%). The concentrations of CH_4, CO_2 and O_2 in the soil were affected by the microbial activity and changes in the load of the gases. The concentration of CH_4 and CO_2 decreased towards the top of the column and there was more variation in the profiles of the treatment column than in the profiles of the control column between different time points (Fig. 3B, C). The concentration of O_2 increased towards the top of the column and there was more O_2 present in the soil of the control column than in the soil of the treatment column (Fig. 3D).

The nutrient content of the soil was measured at each sampling. In the treatment column, the concentration of nitrate increased from 63 to 70 mg kg^{-1} and to 950 mg kg^{-1} following fertilisations and compost amelioration, respectively (Table 3). Similarly, the concentration of phosphate increased from 14.8 to 71 mg kg^{-1} and the concentration of copper increased from 0.114 to 0.270 mg kg^{-1}. Nutrients were not added to the control column during the experiment, and thus the concentration of nitrate decreased from 52 to 22 mg kg^{-1} while the concentration of phosphate and copper remained at approximately at 6.5 and 0.090 mg kg^{-1}, respectively, throughout the experiment.

Table 3
Characteristics of the landfill cover soil, compost and soil-compost mixture in the laboratory columns before the start of the experiment (day 0), after fertilisation or irrigation (day 97), after decompaction (day 120) and after compost addition or decompaction (day 148) (mean ± standard deviation, $n = 1–3$).

Parameter	Cover soil	Compost	Control			Treatment			
Day	0	0	97	120	148	97	120	148	
pH	5.84 ± 0.054	6.87 ± 0.007	6.23 ± 0.041	6.27 ± 0.106	6.31 ± 0.048	6.44 ± 0.083	6.68 ± 0.035	6.91 ± 0.050	
WHC (% dw)	79	218 ± 1.3	79	79	79	79	79	110	
MC (% ww)	33.6 ± 2.17	56.7 ± 2.29	36.8 ± 1.35	33.8 ± 0.30	34.3 ± 1.74	31.8 ± 0.69	35.7 ± 2.11	40.8 ± 2.29	
MC (% WHC)	42.6	26.0	46.6	42.8	43.4	40.2	45.2	37.2	
OM (% dw)	10.4 ± 0.82	41.9 ± 5.30	11.4 ± 0.48	10.1 ± 0.26	10.1 ± 0.70	9.1 ± 0.26	10.7 ± 0.86	17.0 ± 5.30	
NO_3^- (mg kg^{-1})	22.3 ± 1.05	6279 ± 93.9	51.5 ± 5.83	42.6 ± 0.53	22.1 ± 4.77	62.6 ± 18.32	69.5 ± 19.29	950 ± 554.90	
PO_4^{3-} (mg kg^{-1})	14.8 ± 0.77	BD	6.7 ± 1.76	6.9 ± 1.48	6.0 ± 1.15	31.4 ± 9.60	61.2 ± 16.21	71.3 ± 5.61	
SO_4^{2-} (mg kg^{-1})	14.3 ± 0.20	2947 ± 227.5	14.8 ± 3.69	14.9 ± 1.19	18.1 ± 7.75	482 ± 67.3	1092 ± 32.1	1585 ± 123.5	
Cu^{2+} (mg kg^{-1})	0.114 ± 0.057	0.231 ± 0.006	0.028 ± 0.019	0.149 ± 0.001	0.093 ± 0.029	0.193 ± 0.002	0.262 ± 0.008	0.270 ± 0.003	

WHC, water holding capacity; MC, moisture content; OM, organic matter content; dw, dry weight; ww, wet weight; NA, not applicable; n.m., not measured; BD, below limit of detection (<2 mg kg^{-1} dw). The control column was subjected to irrigation and decompaction, and the treatment column to fertilisation and compost addition before sampling at days 97 and 148, respectively
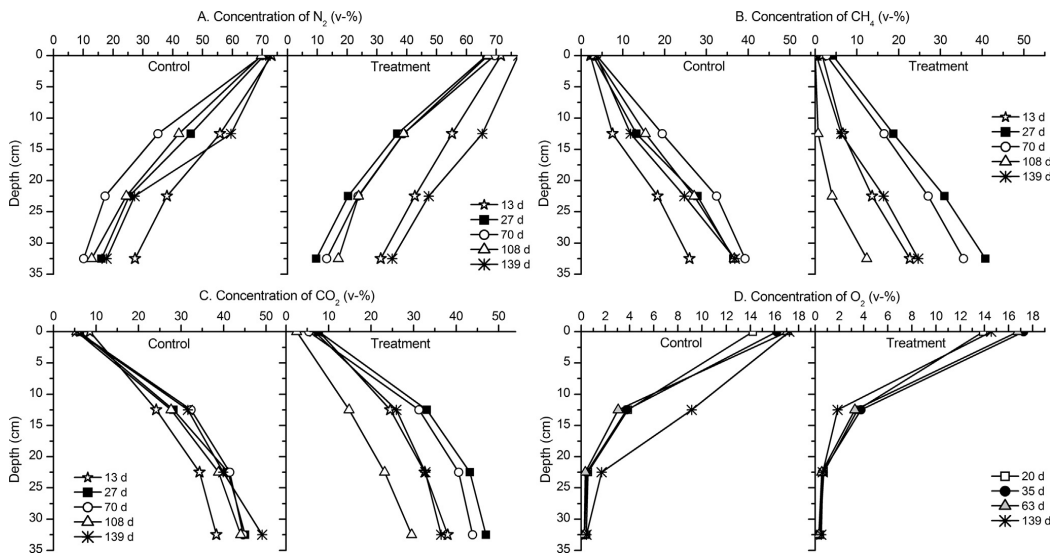
Fig. 3. The depth profiles of N_2, CH_4, CO_2 and O_2 concentrations of control (C) and treatment (T) columns measured before application of any methods (day 13 and 20 at CH_4 load of 110 g m^{-2} d^{-1} and day 27 and 35 at CH_4 load of 210 g m^{-2} d^{-1}), during irrigation (C) or fertilisation (T) and before decompaction (C + T; day 65 and 70), before decompaction (C) or amelioration with compost (T) (day 108), and at the end of the experiment after decompaction (C) or compost addition (T) (day 139).

4. Discussion

In this study, we screened three different methods – inorganic fertilisation, mechanical decompaction and amelioration of the soil with compost – intended to improve the CH_4 EC of mineral landfill cover soil designed for landscaping rather than for CH_4 mitigation purposes. The selected methods targeted two soil characteristics, air capacity and nutrient content, that are crucial for successful CH_4 oxidation activity. The results show that amelioration of soil with compost is superior to the other two methods; fertilisation with inorganic nutrients or decompaction alone did not induce a stable increase in the CH_4 EC of the soil. Optimisation of soil characteristics supporting biological CH_4 oxidation in new biocovers has been studied by many researchers, but to our knowledge, this is the first study aimed at finding solutions to improve the CH_4 EC of existing soil covers that have been in place for several years.

4.1. CH_4 elimination capacity of the landfill soil

The mineral landfill soil (silty gravelly sand) studied here was shown to support methanotrophic activity; however, this activity was not sufficient to deal with the typical CH_4 load generated by closed landfills; the average CH_4 EC obtained for both columns prior to any treatments was 50 g CH_4 m^{-2} d^{-1}, or 24% removal of added CH_4. This value represents the lower range of CH_4 ECs reported for mineral landfill soil in other column studies, which range from 61 to 71 g m^{-2} d^{-1} (37–43% RE) (Huber-Humer et al., 2011) to 152–159 g m^{-2} d^{-1} (95–99% RE) (De Visscher et al., 1999) (Table 4). As the soil here had higher moisture content, WHC, organic matter content, and lower bulk density – characteristics that are shown to support methanotrophic activity – than the soils used in previous studies, the lower CH_4 EC may be explained by other factors as discussed later.

Table 4
Comparison of CH$_4$ elimination capacities and removal efficiencies for column setups simulating performance of landfill covers with passive aeration.

Material	Soil type	Improvement	MC (% ww)	WHC (% dw)	OM (% dw)	Bulk density[a] (kg L^{-1})	Inlet load (g m^{-2} d^{-1})	EC (g m^{-2} d^{-1})	RE (%)	Ref.
Landfill soil	Sandy loamy soil	–	16–25	31–41	3.6–5.2	1.26–1.35*	165	61–71	37–43	[1]
Landfill soil	Sandy loamy soil (60% sand, 31.6% silt, 8.4% clay)	–	14	n.r.	1.7 (carbon)	0.96–1.04	160	152–159	95–99	[2]
Landfill soil	Silty gravelly sand (39.8% gravel, 49.9% sand, 10.3% silt)	–	34	79	10.4	0.87	370 / 110 / 210	290 / 74[d] / 52[d]	68 / 27	[7]
Soil	Loamy sand	44, 111 or 221 mg NO$_3^-$ kg^{-1} (3[b]) / +80 mg P kg^{-1} (21[b]) / +150 mg K kg^{-1} (40[b])	13	n.r.	0.4	1.61	526	428[(44 mg NO3-)] / 436[(111 mg NO3-)] / 432[(221 mg NO3-)]	81 / 83 / 82	[3]
Landfill soil	Coarse sand (70% coarse sand, 18% fine sand, 16% silt/clay)	Control / 2000 mg N kg^{-1} as NH$_4$NO$_3$ (n.r.[b]) / 100 mg P kg^{-1} as K$_2$HPO$_4$ (n.r.[b]) / 100 mg P + 100 mg N kg^{-1} as SS (n.r.[b])	11 ±7	55	~0.8	n.r.	271	92[(Control)] / 33[(NH4NO3)] / 92[(K2HPO4)] / 116[(SS)]	34 / 12 / 34 / 43	[4]
Landfill soil	Silty gravelly sand (39.8% gravel, 49.9% sand, 10.3% silt)	637 mg NO$_3^-$ kg^{-1} (22[b]) / 425 mg PO$_4^{3-}$ kg^{-1} (15[b]) / 0.528 mg Cu^{2+} kg^{-1} (0.11[b]) / 1180 mg SO$_4^{2-}$ kg^{-1} (14[b])	34	79	9.9	0.89	210	87[e] (45–203)	41[e] (21–100)	[7]
Leaf compost	Particle size < 5 mm	Decompaction	55	n.r.	46	0.64*	520	100 → 500	19 → 95	[5]
Soil	Silty gravelly sand (39.8% gravel, 49.9% silt)	Decompaction	35	79	10.5	0.83	210	47[f]	22[f]	[7]
Landfill soil	Silty clayey soil (30% sand, 30% silt, 40% clay) + compost (<10 mm)	Compost 25 w-% / Compost 50 w-%	19 / 22	n.r.	n.r.	1.39 / 1.21	680	137 / 175	20 / 26	[6][g]
Garden soil	Soil type n.r. + compost (<20 mm or <15 mm)	Compost 60 w-%	30–47	60–79	14–16	1.14–1.20*	165	149–165	90–100	[1]
Landfill soil	Silty gravelly sand (39.8% gravel, 49.9% sand, 10.3% silt) + compost (<20 mm)	Compost 22 w-%	41	110	17.6	0.64	210	189[c]	88	[7]

MC, moisture content; dw, dry weight; ww, wet weight; WHC, water holding capacity; OM, organic matter; EC, elimination capacity; RE, removal efficiency; n.r., not reported; SS, sewage sludge; w-%, percentage by weight.
References: [1] Huber-Humer et al. (2011), [2] De Visscher et al. (1999), [3] Park et al. (2002), [4] Kightley et al. (1995), [5] Wilshusen et al. (2004), [6] Rose et al. (2012), [7] this study.

[a] Bulk densities are expressed relative to dry mass of soil, except for (*) where the parameter was not specified by the authors (wet or dry density).
[b] Initial nutrient concentration in the soil prior to fertilisation (mg kg^{-1}).
[c] Calculated from data obtained with the treatment column between days 120 and 148.
[d] Calculated from data obtained with the treatment column between days 120 and 148.
[e] Calculated as an average value from data obtained with the control and treatment columns between days 0–24 and 25–64.
[e] Calculated from data obtained from the treatment column between days 65 and 120, which includes two instances of fertilisation; average, minimum and maximum values are presented. Data range includes one decompaction treatment (at day 97), the effect on CH$_4$ consumption of which was shown to be insignificant.
[f] Calculated from data obtained with control column between days 98 and 148, which includes one irrigation treatment (at day 120). Its effect on CH$_4$ elimination was shown to be negligible.
[g] Active aeration from the bottom of the column.

The factor limiting the activity of methanotrophs in the landfill soil studied here was so dominant that even increasing the supply of CH_4 and availability of O_2, the factors believed to be the most essential for methanotrophic activity (e.g. Bohn et al., 2011; Scheutz et al., 2009), did not enhance the CH_4 elimination capacity of the soil. The CH_4 load was doubled in the beginning of the experiment, offering more substrates to the methanotrophs, but the CH_4 EC of neither column followed expected patterns (e.g. Einola et al., 2008). Increases in CH_4 EC have been reported to result from the enrichment of methanotrophs as a response to increased CH_4 load, but here the number of methanotrophs and the average MOP of the landfill soil (as shown by the control column) remained relatively constant throughout the experiment, in agreement with the stable CH_4 RE. The CH_4 load was increased by increasing the flow rate of CH_4 from the bottom of the column, which slightly hindered penetration of N_2 (and O_2) from the top of the column. It is possible that the decreased supply of O_2 could slow methanotrophic activity in the columns, but this was disproved based on the treatment column, whose CH_4 RE increased following application of different methods. Thus, some other factor than availability of CH_4 and O_2 were limiting the activity of methanotrophs.

4.2. Effect of fertilisation with inorganic nutrients

Methanotrophs in the studied landfill soil benefitted from soil fertilisation with inorganic nutrients, after which an increase in CH_4 consumption was noted, but the effect was short-lasting. The studied landfill soil was depleted of nutrients (Table 3) and probably did not meet the nutritional requirements of methanotrophs (0.12 g nitrogen per 1 g oxidised CH_4; total phosphorus >0.3% dw, calculated from phosphate $\geq$55 mg kg^{-1}; sulphate >500 mg kg^{-1}; copper >0.50 mg L^{-1} of soil water) (De Visscher et al., 1999; Huber-Humer et al., 2009; Maanoja and Rintala, 2015; van der Ha et al., 2010). Consequently, the nutrients were quickly used up by the bacteria following fertilisation, as indicated by the disappearance of the added nutrients by the next sampling. Fertilisation induced an increase in the number of cultivable methanotrophs, which reflected the improved MOP of the soil, but the CH_4 EC increased for only a short period, suggesting that the demand of methanotrophs for nutrients exceeded the amount of added nutrients.

The design of this study does not allow us to distinguish which nutrient caused the increase in CH_4 EC, but it seems that the effect of inorganic fertilisation is rather unpredictable. In our study, the main nutrients consumed by the bacteria were nitrate, phosphate and copper, but the literature describes rather contradictory responses of methanotrophs to the nutrients in landfill soil. An addition of 8850 mg NO$_3^-$ kg^{-1} as NH$_4$NO$_3$ was reported to decrease CH_4 EC (Kightley et al., 1995) and an addition of 44–220 mg NO$_3^-$ kg^{-1} was reported to have no effect on CH_4 EC (Park et al., 2002) (Table 4), while MOP has been measured to be higher in soil materials containing approximately 1000 mg NO$_3^-$ kg^{-1} than in soils containing 250 mg NO$_3^-$ kg^{-1} (Huber-Humer et al., 2011). For phosphate, additions show either no effect on CH_4 oxidation during a column experiment (80–100 mg P kg^{-1} soil added as K$_2$HPO$_4$) (Kightley et al., 1995; Park et al., 2002) or some effect during a batch experiment (18–60 mg P kg^{-1} added as an NPK fertiliser) (Jugnia et al., 2012). One explanation for these discrepancies may be that the response of different types of methanotrophs varies between nutrients, and hence, the composition of the community present in the soil governs the effect of fertilisation on CH_4 oxidation (Veraart et al., 2015). If this is the case, fertilisation of landfill cover appears to be an inefficient method for improving the CH_4 EC because its effect on different landfills cannot be easily foreseen without preliminary laboratory testing.

4.3. Effect of soil decompaction by mixing

Contrary to expectations, the gas conduction properties of the mineral landfill soil did not deteriorate during the experiment; thus, decompaction was not effective insofar as there was no lost air capacity of the soil to restore. The hypothesis was that the fine-grained inorganic landfill soil (approximately 50% of particles <2 mm) would become compacted or the gas pores blocked by EPS, thus restricting the diffusion of gases to methanotrophs, and that decompaction could subsequently improve the conditions. We did not measure the production of EPS, but clear orange-red bacterial zones were visible in the topmost soil of both columns. These layers have been shown to contain a high number of methanotrophic cells (Humer and Lechner, 1999) and a high concentration of EPS (Mancebo and Hettiaratchi, 2015; Wilshusen et al., 2004). Herein, the accumulation of methanotrophs in the topmost orange-coloured soil was confirmed by the MOPs, which were higher in the topmost soil than at the bottom of the column. Despite the formation and disappearance of the putative EPS, the diffusion of N_2 (or O_2) did not significantly decrease in the columns prior to decompaction and did not improve afterwards, indicating that the soil structure did not deteriorate during the experiment.

Even though decompaction of the soil did not affect the diffusion of gases, it did help to distribute the colonised methanotrophs and homogenise the soil bed. During mixing of the soil, the orange-red biofilm disappeared and the cells from each zone were distributed evenly throughout the soil, improving the opportunity for each methanotrophic cell to encounter a CH_4 molecule. After decompaction by mixing, the CH_4 EC did peak shortly in the treatment column, which had also received inorganic nutrients, whereas in the control column, decompaction seemed to disturb the methanotrophic community and the CH_4 EC temporarily decreased. We can only speculate the reason for this, but it is likely that the previously added nutrients contributed to the increase in CH_4 EC in the treatment column. Potentially, the methanotrophs in the treatment column accumulated nutrients in their biofilm of EPS (Mancebo and Hettiaratchi, 2015) or the nutrients remained unused in the portion of the soil bed in which the bacterial density was lower. Mixing of soil could have better distributed these nutrients throughout the soil, making them available to the methanotrophs.

4.4. Effect of soil amelioration with compost

The highest CH_4 EC obtained in this study, 208 g m^{-2} d^{-1} (189 g m^{-2} d^{-1} on average), was reached after amending the soil with compost; at this level, there was almost complete removal of the inserted CH_4 (RE 88% on average, 95% at maximum). The CH_4 EC was slightly higher than reported for different soil and compost mixtures presumably because of the different physical and chemical properties of the amended soil (Table 4): 149–165 and 137–175 g m^{-2} d^{-1} (RE 90–100% and 20–26%, respectively) (Huber-Humer et al., 2011; Rose et al., 2012). One finding of interest for practical applications was that the modest substitution of 22 w-% of the soil with compost was sufficient to increase the CH_4 EC of the soil to a level corresponding to the typical CH_4 production rate of a municipal solid waste landfill (132–232 g m^{-2} d^{-1}) (Huber-Humer, 2004). Effectiveness of a modest compost addition in increasing the soil CH_4 EC was also shown by Wei et al. (2016), who showed that the same CH_4 oxidation potential can be achieved by additions of both 20 and 50 w-% composted cattle manure if the exposure to CH_4 is continuous. However, their study was conducted as a batch experiment. An earlier column study (Rose et al., 2012), suggested 50 w-% as the minimum addition for significantly increasing the CH_4 EC. However, it might be that addition of a larger amount of compost has more long-

lasting effects on the soil properties and thus on CH_4 oxidation than smaller additions.

The increase in the CH_4 EC of the soil after addition of compost can be attributed to the improved diffusion of gases in the soil due to higher total porosity and, more importantly, to higher WHC of the soil-compost mixture. Diffusion of gases in the treatment column improved considerably after addition of compost (Fig. 3); the low dry bulk density of the pure compost decreased the bulk density of the soil, creating more pores for water and air. Most of these pores, however, remained filled with water, and the AFPS was similar to that of the control soil (0.24 vs. 0.23 L L^{-1}) due to the high water content of the compost. This indicates that the increased CH_4 EC following from improved diffusion of gases could not result from greater AFPS of the soil; instead, it is likely due to the increased WHC of the soil-compost mixture. The reason for this could be that high soil WHC is associated with high organic matter content, uniform particle size distribution and loose arrangement of particles (high porosity), all of which enable the soil to retain large amounts of water relative to the dry mass while leaving some of the pores unsaturated, which facilitates diffusion of gases through the material (Bohn et al., 2011; Olorunfemi et al., 2016). It should be remembered, however, that the soil studied here was coarse silty sand and the effect of compost on the gas diffusion properties, such as permeability and porosity, and CH_4 EC of finer and loamy soil might be less pronounced (Rose et al., 2012).

The increase in the CH_4 EC of the treatment column was undeniably supported by the great increase in the number of cultivable methanotrophs (from 13 to 705 × 10^6 cells g^{-1}) and the increased concentration of nutrients in the soil after addition of compost. The significance of compost amendments, organic nutrients and additional bacteria to the activity of methanotrophs cannot be separately judged from the data obtained herein, and it is possible that they had a combined effect. Earlier studies have shown, however, that organic nutrients are released from the organic compounds of compost slowly via decomposition processes (Huber-Humer et al., 2011) and that organic fertilisation accelerates while inorganic fertilisation hinders CH_4 oxidation activity in soil relative to untreated soil in the long term (Seghers et al., 2003). As for the methanotrophic population of the treatment column, it originated either from external methanotrophs in the compost (29.6 × 10^6 cells g^{-1}) or from the multiplication of the indigenous soil methanotrophs after improvement of the growth conditions – gas diffusion, specific surface area and nutrient content – in the soil-compost column. The origin of methanotrophs in compost-amended soil has also puzzled other researchers (Seghers et al., 2003), but nevertheless, amelioration of the soil with compost brought on an increase in the number of methanotrophs.

Addition of compost increased the basal respiration activity and consumption of O_2 in the landfill soil, but the heterotrophic bacteria could not outcompete the methanotrophs. This was achieved by using compost that had gone through a full stabilisation process over 13–24 months and exhibited organic matter content of 41.9% dw and respiration activity of 2.74 mg O_2 per gram of soil calculated from the CO_2 production rate of heterotrophic bacteria (22.5 μg CO_2 g^{-1} h^{-1} at 22 ± 2 °C) assuming a period of seven days and molar ratio of 1:1 O_2:CO_2 (RA_7). The maturity of the compost is crucial for the success of compost amendment because in immature compost (e.g. $RA_7 \geq 8$ mg O_2 g of $soil^{-1}$; Huber-Humer et al., 2009), the heterotrophic bacteria that degrade the organic matter compete with methanotrophs for nutrients and O_2, which might result in decreased CH_4 oxidation activity (Huber-Humer et al., 2011). Here, methanotrophs dominated the microbial activity in both columns throughout the experiment, including after addition of compost to the treatment column, as shown by the CO_2 production rates measured in the batch experiment (contribution of methanotrophs was 56.2% on average at day 148) and the molar ratios of CO_2 produced to CH_4 oxidised determined from the columns (0.63 on average after compost addition).

All three methods of improving soil CH_4 EC were applied to one soil batch in the treatment column sequentially, so possible additive effects cannot be completely excluded. It is possible that the microbial community and, by implication, their metabolic activity could have changed because of inorganic fertilisation relative to the untreated soil. We did not analyse the microbial community in this study, but a field experiment showed that inorganic fertilisation (NH_4NO_3, P_2O_5, K_2O) inhibits the activity of methanotrophs rather than alters the composition of their community (Seghers et al., 2003). Here, fertilisation had a more accelerative effect than an inhibitory effect. The concentration of nutrients added to the soil by compost was significantly higher compared to the amount added by fertilisation apart from phosphate, as the compost contained very little phosphate. Thus, it is possible that the sequentially applied methods had a minor additive effect on the activity of methanotrophs in the soil of the treatment column, but the benefits of the compost amendment were so great that the outcome would probably have been the same even without the earlier treatments.

The present results suggest that, for example, in the studied Tarastenjärvi landfill, where approximately 10.3 g CH_4 m^{-2} d^{-1} was measured to escape from the landscaped soil cover of the old part of the landfill in July 2013 (Pohl and Kuuri, 2013), the CH_4 EC of the soil cover could be improved by the addition of 22 w-% of compost. In a landfill cover of 10 ha, this requires ameliorating the soil (30,000 m^3 or 31,320,000 kg dw calculated with bulk density of 1305 kg m^{-3} (Huber-Humer et al., 2011) and moisture content of 20% ww; Table 4) with 17,670,000 kg ww of compost (moisture content 50% ww) if we assume that the mixing is carried out by ploughing to a depth of 0.3 m. In our experiment, the CH_4 EC of the soil was increased by 55.3 g m^{-2} d^{-1} by the compost amelioration, so it is reasonable to expect that the aforementioned CH_4 emissions from Tarastenjärvi landfill could be significantly reduced by the treatment on a simplified average level without taking into account seasonal variation. The other methods screened here – inorganic fertilisation and decompaction by ploughing – would not be effective.

5. Conclusions

Our aim was to study the feasibility of different methods (inorganic fertilisation, decompaction and amelioration with compost) for improving the CH_4 elimination capacity of mineral landfill soil (organic matter 10% dw) consisting of nutrient-poor silty gravelly sand, thereby providing novel information on ways to control CH_4 emissions in old landfills. The studied landfill cover soil did not exhibit high enough values for parameters such as porosity, WHC and nutrient content that support sufficient CH_4 oxidation activity for mitigating emitted CH_4. The aforementioned properties and CH_4 elimination capacity of the soil were improved by addition of compost (22 w-%) to the soil, which created conditions for proliferation of methanotrophs. Fertilising the landfill soil with inorganic nutrients increased the nutrient concentration in the soil and met the demand of methanotrophs for nutrients, but the access of the bacteria to the nutrients might be incomplete without mechanical mixing of the soil. Decompaction alone proved the least effective method for improving the soil, as the well-graded medium coarse mineral soil did not seem to become compacted or clogged, which would have prevented diffusion of gases, during the time span of the experiment. Therefore, we suggest that amelioration of the soil with compost could be utilised to treat post-closure emissions of CH_4 from old landfills after years of operation, while inorganic fertilisation and decompaction appear unjustified

for practical applications on landfills consisting of soil similar to that studied here.

Declarations of interest

None.

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PUBLICATION
III

Compacted bentonite as a source of substrates for sulfate-reducing microorganisms in a simulated excavation-damaged zone of a spent nuclear fuel repository

Maanoja, S., Lakaniemi, A., Lehtinen, L., Salminen, L., Auvinen, H., Kokko, M., Palmroth, M., Muuri, E. & Rintala, J.

Research Paper

Compacted bentonite as a source of substrates for sulfate-reducing microorganisms in a simulated excavation-damaged zone of a spent nuclear fuel repository

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A B S T R A C T

Sulfide formed by sulfate-reducing microorganisms (SRM) is a potential safety risk in the geological disposal of spent nuclear fuel (SNF) enclosed in copper canisters because it can corrode copper. The canisters will be isolated from the environment by surrounding them with compacted bentonite. This study shows experimentally that the organic matter naturally present in compacted bentonites can become dissolved and sustain biological sulfate reduction. The experiment was conducted in cell systems consisting of an interface of compacted bentonite (at dry density of 1314–1368 kg m^{-3}) and a low-porosity sand layer representing an excavation-damaged zone of the host rock. Some cells were inoculated with SRM and groundwater microorganisms and some were not. Varying concentrations of organic matter and sulfate in the sand layer solution resulted from partial dissolution of the studied bentonites (Wyoming, Indian, and Bulgarian). The dissolved organic matter promoted biological sulfate reduction, as demonstrated by the decrease in sulfate concentration in the sand layer solution and the formation of sulfide iron precipitates in the inoculated cells relative to the uninoculated cells. Other anaerobic microorganisms (e.g., methanogens) also became active in the cells and they along with the SRM were found to grow within the sand and/or bentonite layers of the cells. The findings of this study show that bentonites can sustain biological sulfate reduction in areas with lower density and immobilize possibly formed sulfides. However, the extent of these capabilities seems to be affected by the mineralogy of bentonites in the studied density range.

1. Introduction

One of the management options for spent nuclear fuel (SNF) generated by the nuclear power industry is direct disposal in geologic repositories. For example in Finland, the disposal of SNF to the Olkiluoto bedrock is planned to be initiated in the 2020s (Posiva, 2018). According to the plan, the SNF will be disposed of in deposition holes drilled along the tunnels of the underground repository and isolated from the surrounding environment by an engineered barrier system. The barrier system will consist of a copper/iron canister designed to contain the SNF, buffer bentonite to protect the canister in the deposition hole, and backfill bentonite to seal the tunnels leading to the holes (Posiva, 2012a). Based on the expected decay of the radioactivity of the SNF, the repository should provide passive safety for hundreds of thousands of years (Posiva, 2012b). One factor that can possibly affect canister integrity is biologically produced sulfide as sulfide is a well-known corroding substance for copper (King et al., 2012).

Ubiquitous in nature, anaerobic sulfate-reducing microorganisms (SRM) including both bacterial and archaeal species have been found in the bedrock and groundwater of Olkiluoto (Muyzer & Stams, 2008; Pedersen et al., 2014). While reducing sulfate to sulfide, SRM use organic compounds, CO_2 and/or H_2 to obtain energy and carbon for growth (Liamleam & Annachhatre, 2007; Muyzer & Stams, 2008). The growth of SRM in bedrock and groundwater is limited by the low availability of organic matter (Wolfaardt & Korber, 2012; Rajala et al., 2015). However, as a result of the final disposal of SNF, the buffer and backfill bentonites will introduce organic matter (Hallbeck, 2010), which could serve as a substrate for microorganisms in the repository.

The organic matter in bentonite is chemically recalcitrant and contains long-chain and aromatic compounds (Durce et al., 2015; Marshall et al., 2015), which are assumed to be thermodynamically unfavorable substrates for SRM (Cassidy et al., 2015). However, when SRM live in a diverse microbial community, such as that typically present in groundwater (e.g., Bomberg et al., 2015) or bentonites (Masurat et al., 2010; Stone et al., 2016), hydrolytic and fermentative microorganisms can degrade the complex organic compounds into a more utilizable form for the SRM (Zavarzin et al., 2008). The ability of SRM to grow on the organic matter of bentonite has been hypothesized (Bengtsson et al., 2017) but never demonstrated experimentally.

One function of the bentonite buffer is to limit microbial activity in the near field of the SNF canisters and retard the migration of possibly formed sulfides to the canisters (Posiva, 2012a). Earlier studies suggest that this function can be achieved by compacting the buffer bentonite to a high dry density (≥ 1370 kg m^{-3}, corresponding to a wet density of 1880 kg m^{-3}; Bengtsson & Pedersen, 2017). In a compacted buffer, low porosity hinders the dissolution of the complex organic matter from bentonite to water (Durce et al., 2015), and low water activity ($a_w < 0.96$) suppresses the activity of microorganisms (Stroes-Gascoyne et al., 2010). The repository, however, will feature lower density areas where the microbial activity and sulfide formation can occur (Stroes-Gascoyne et al., 2011). These areas include interfaces of the buffer and backfill with the host rock, particularly in the micro-fractures in excavation-damaged zones (EDZs) of the tunnels, where the groundwater can more readily interact with the bentonite and cause the dissolution of ions and organic matter from the bentonite (Stroes-Gascoyne et al., 2011; Wolfaardt & Korber, 2012).

This work aimed to study experimentally whether organic matter can dissolve from compacted bentonite and sustain biological sulfate reduction in cell systems that mimic the interface of the host rock and the compacted bentonite in an EDZ of a SNF repository. Quartz sand was used as a material conservatively mimicking the conditions in a lower density area (such as the EDZ), where the hydraulic conductivity and porosity are considerably higher than in intact rock (Posiva, 2013). In the experiment, two possible sources for microbial growth into the EDZ were studied: external, a groundwater-originated community enriched with known SRM, and indigenous, a bentonite-originated community. Three bentonites from different origins (Wyoming, India, and Bulgaria) were tested as an example of materials that could be used as part of the engineered barrier system for a geologic repository for SNF. The characteristics of Wyoming and Indian bentonites, including their physical, chemical, and mineralogical properties, hydromechanical behavior in repository-related conditions, and threshold density for suppressing microbial activity, have been studied extensively (e.g., Rautioaho & Korkiala-Tanttu, 2009; Kiviranta & Kumpulainen, 2011; Bengtsson & Pedersen, 2017; Cui, 2017), whereas the corresponding characteristics of Bulgarian bentonite have been studied less. To the authors' knowledge, the possible effect of the three bentonites on microbial activity by acting as a potential source of organic matter has never been studied.

2. Materials and methods

The experiment was carried out in six uniquely designed experimental cells having two sections, which consisted of a layer of saturated compacted bentonite and a layer of loosely packed quartz sand (Fig. 1). Cells were prepared by using three different bentonites and the preparation steps included saturation and compaction of the bentonite blocks (described in Chapters 2.1 and 2.2). Furthermore, the blocks were re-compacted twice at the later stages of the experiment (Chapter 2.2). The sand layers were assembled on top of the compacted bentonite blocks, and the sand layer of one cell of each bentonite was inoculated with a mixture of known SRM and microorganisms enriched from Olkiluoto groundwater (Chapter 2.3). These cells were referred to as "inoculated cells." The sand layer of the other cell of each bentonite was

not inoculated with external microorganisms, but as the used bentonites were not sterilized, the presence of microorganisms indigenous to bentonites was anticipated. These cells were referred to as "uninoculated cells."

2.1. Bentonites, quartz sand, and artificial groundwater

The bentonites used in the experiment included two sodium bentonites from Wyoming and India and one calcium bentonite from Bulgaria (Table 1). The Indian and Bulgarian bentonites (grain size 0.5–3 mm) were ground to ensure homogeneity (100% < 0.2 mm), while the Wyoming bentonite was used as is because it was originally finer material than the other bentonites (68% ≤ 0.5 mm; Kiviranta & Kumpulainen, 2011). After this, the bentonites were stored in sealed plastic bags in aerobic conditions at 6 °C. The quartz sand (NFQ Nilsiä QUARTZ, Sibelco Nordic Oy Ab, grain size of $0.63 \leq x < 1$ mm) was combusted (4 h at 450 °C) to remove organic residues. Prior to the experiment, O_2 was removed from the bentonites and sand by purging them with N_2 in a desiccator as follows; the desiccator holding the bentonite or sand was first evacuated (10 min, < 10 mbar) and then filled with N_2 (99.5% v/v, Aga Ltd.). These steps were repeated four more times after minimum of 20 min of equilibration time as determined in preliminary testing. After deoxygenation, all the following preparation steps (i.e. saturation and wetting) took place in an anaerobic hood (Whitley A35 Anaerobic Workstation, 100% v/v N_2).

Artificial groundwater (AGW) with controlled concentration of dissolved organic carbon (DOC; < 0.2 mg L^{-1}) was used to saturate the bentonites and sand in the cells. Composition of the AGW corresponded to a defined reference water expected to occur at the Olkiluoto repository: 6.65 g NaCl, 4.77 g CaCl$_2$·2H$_2$O, 519 mg MgCl$_2$·6H$_2$O, 56.7 mg NaBr, 42.6 mg SrCl$_2$·6H$_2$O, 29.6 mg Na$_2$SO$_4$, 21.0 mg KCl, 7.4 mg H$_3$BO$_3$, 2.2 mg NaF, and 0.03 mg NH$_4$Cl in 1 L ultrapure water (Milli-Q®, MilliporeSigma; pH 6.5; modified from Hellä et al., 2014).

2.2. Cell design and preparation of the bentonite blocks

The cells (Fig. 1) were made of polyether ether ketone with high chemical resistance and low reactivity and stainless steel (316-L) with high mechanical strength. In the cells, the bentonite layer (5.0 L) located at the bottom, and the sand layer was assembled on top of the bentonite inside a separate sleeve (662 mL). A porous titanium sinter (pore size 1–2 μm; GKN Sinter Metals GmbH; purified with 8% w/v HNO$_3$ and ultrapure water) separating the bentonite and sand allowed the migration of ions, molecules and microbial cells between the layers. The O-rings used for sealing the cells were inert and had low gas permeability (Viton®). Microvalves (VICI AG) were attached to the plunger for sampling and balancing the pressure during compaction. Organic residues were removed from the parts by washing with propanol, 1 M HCl or AGW, and ultrapure water.

Preparation of the bentonite blocks was started by saturating and compacting the bentonites by using the method described by Herbert et al. (2008) as follows. First, batches of deoxygenated bentonite and degassed AGW (≥ 30 min with N_2) required for preparing saturated bentonite blocks (Eqs. S1–S5; Chapter S1; Table 2) were mixed. Then, the mixture was allowed to wet for 1–4 days before compaction, except for some of the mixtures of Indian and Wyoming bentonites for the uninoculated cells, which wetted for 20 days due to the unexpected additional adjustments required for these cells. If any organic matter dissolved in AGW from the bentonite during the wetting period, it became compacted inside the bentonite block. After wetting, the bentonites were compacted to the target dry density of 1400 kg m^{-3}, which results in a target swelling pressure currently assumed to suppress activity of microorganisms in Wyoming type bentonite (approximately 2 MPa; Kaufhold et al., 2015; Taborowski et al., 2019). The bentonite blocks were compacted in four layers (4 cm each) to obtain as homogeneous density as possible throughout the blocks. Compaction (with
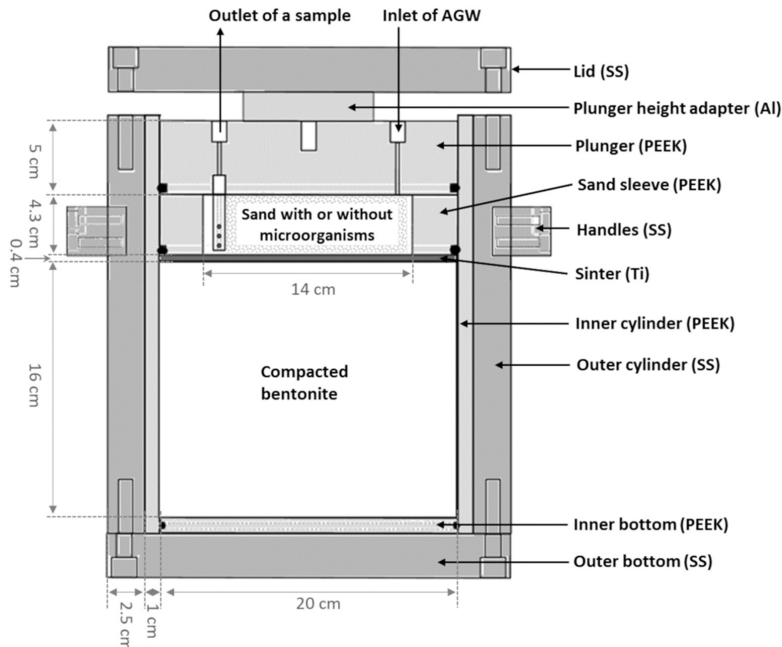
Fig. 1. Schematic of the experimental cell (dimensions are in scale; AGW, artificial groundwater; SS, stainless steel; PEEK, polyether ether ketone).

hydraulic pressure of 20 MPa) took 2–192 h for each layer to reach the target height and, thus, density calculated according to Eq. S1 (Table 2). A sinter saturated with AGW was pressed on top of the compacted bentonite block, and AGW was added on the sinter to prevent it from desaturation (50–65 mL, until the sinter was soaked).

After completing the initial compaction of all six bentonite blocks one after another (hence stored for 10–108 days), re-compaction of the blocks was needed as it was noticed that the unconstrained bentonite blocks had swelled during storage (by 61–326 mL, to a dry density of 1302–1380 kg m^{-3}; details given in Chapter S2.1, Table S1). Thus, all the bentonite blocks were re-compacted to the original target density (Table 2). The changes in sulfate and DOC contents of all bentonite blocks caused by swelling and re-compaction were < 0.2% (w/w) of the initial contents except for Bulgarian bentonite in the uninoculated cell, where the decrease in DOC content was 14% (w/w) (Chapter S2.2, Tables S2, S3). The change in that block was greater than in the others because the assemblance of the sand layers was started with the uninoculated cell of the Bulgarian bentonite (as described in Chapter 2.3), and the bentonite adsorbed the AGW added both on the sinter and in the sand sleeve before it was noticed that re-compaction was required (Chapter S2.1).

Another re-compaction was required during the experiment (at operational days 146 and 167) due to swelling of bentonites inside the cells (by 184–328 mL, to a dry density of 1309–1337 kg m^{-3}; Table S1), which resulted from breakage of the plunger height adapters (Fig. 1). The bentonite blocks were re-compacted to dry densities of 1314–1369 kg m^{-3} (Table 2) and the cells were equipped with new plunger height adapters. The swelling and re-compaction of bentonite did not cause a release of sulfate or other inorganic compounds from the bentonites (Chapter 3.2; no anomalies in data at days 146 and 167). The implications of swelling and re-compaction on the bentonite densities (decrease of 6% in the uninoculated cell of the Bulgarian bentonite, 3% on average in the other cells in comparison to the density in

the beginning of the experiment) and increased release of DOC are further discussed in Chapters 3.1 and 3.4.

2.3. Preparation of the sand layers and start-up of the experiment

To start the experiment, the sand layers were assembled for the uninoculated and inoculated cells separately. For the uninoculated cells, combusted, uninoculated sand, and unsterilized, uninoculated AGW deoxygenated with sterile-filtered N$_2$ (0.2 μm) were used (Table 2).

For the inoculated cells, the sand and AGW were both amended with microorganisms. The sand was mixed with groundwater from Olkiluoto (drill hole ONK-PVA06), and the groundwater microorganisms were fed sequentially with leachates of bentonites in a pre-enrichment step described in Chapter S3.1. The AGW was amended with microorganisms from three sources: 1) pure cultures of SRM (*Desulfobacula phenolica*, *Desulfobulbus mediterraneus*, *Desulfobulbus rhabdoformis*, *Pseudodesulfovibrio aespoeensis*, and *Desulfotomaculum acetoxidans* from DSMZ GmbH) previously identified from Olkiluoto groundwater (Pedersen et al., 2014; Bomberg et al., 2015; Rajala et al., 2015), 2) microorganisms present in fresh Olkiluoto groundwater (ONK-PVA06), and 3) pre-enriched pelagic microorganisms not attached to the sand during the pre-enrichment step (details given in Chapter S3.2). The AGW and sand amended with microorganisms were inserted to the inoculated cells (Table 2).

Samples were taken from the uninoculated and inoculated sand and AGW to determine the initial number of microorganisms (SRM and total bacteria) and overall microbial activity in the sand.

2.4. Cell operation, monitoring and sampling

After the sand layers were assembled, the cells were closed with plungers and a sealant (Sikaflex®-11FC, Oy Sika Finland Ab). The

Table 1

Mineral composition and major elements as oxides of Wyoming, Indian, and Bulgarian bentonites expressed as mass-% of the dry material.

Bentonite	Wyoming[a]	Indian[b]	Bulgarian[c]
Minerals			
Smectite	88	74	68
Illite	< 1	1	7
Calcite	< 1	9	11
Gypsum	< 1	1	< 1
Plagioclase	3	tr.	< 1
Pyrite	1	0	0
Other	8	15	14
Elements			
Sulfate-S	0.12	0.06	0.05
Sulfide-S	0.15	< 0.02	< 0.02
Fe^{3+}	2.1	10.6	3.2
Fe^{2+}	0.57	0.05	0.26
Inorganic C	0.15	0.36	n.r.
Organic C	0.15	0.15	0.11
Total C	n.r.	n.r.	0.87
LOI	6.3	12	17
CEC	863	850	700
Exchangeable cations			
Na	580	450	60
K	20	0	20
Ca	240	270	450
Mg	90	160	100
SiO_2	62	45	53
Al_2O_3	21	19	14
Fe_2O_3	3.9	15	5.0
FeO	0.53	n.r.	n.r.
TiO_2	0.17	1.0	0.82
MgO	2.5	2.5	2.8
CaO	1.3	3.0	5.7
Na_2O	2.4	1.5	0.34
K_2O	0.78	0.10	1.2
P_2O_5	0	0.10	0.36
Cr_2O_3	0	0.036	< 0.002
MnO	0	0.13	0.10

n.r., not reported; tr., trace amount; CEC, cation exchange capacity and exchangeable cations (equivalent g^{-1}); LOI, loss on ignition.

[a] Kiviranta & Kumpulainen, 2011; Kiviranta et al., 2018.
[b] Kumpulainen & Kiviranta, 2015.
[c] Kumpulainen et al., 2016.

following day, AGW (16–33 mL) was added in the sand layer through the sampling valve to ensure saturation of the sand. Shortly after, the first sample (11 mL; denoted as day 0) was collected from the sand layer solution by a gas-tight glass syringe (VICI AG). The sampled volume was optimized with respect to the volume of the sand layer solution (3% v/v) to minimize perturbation of the sand layer solution during sampling. A corresponding volume of fresh AGW was inserted to the sand layer while the sample was extracted. All the following samplings were carried out in a similar manner unless stated otherwise.

The sand layer solution was sampled at days 0, 14, and 21 and then every three weeks until days 370–454, when the cells were terminated and processed for sampling one after another. The dissolution of bentonite constituents to the sand solution was monitored by measuring the concentrations of DOC and dissolved inorganic carbon (DIC), sulfide, sulfate, and total iron and the redox potential (E_h) and pH of the sand layer solution. Only E_h was measured from the samples taken from the cells of the Indian bentonite between days 43 and 85 because before analyzing the other parameters, the E_h was anticipated to decrease < –50 mV (vs. standard hydrogen electrode, SHE) for enabling the activity of SRM similarly as occurred in the other inoculated cells. As the E_h did not drop below –50 mV in the cells of the Indian bentonite, measurement of other parameters was continued from day 106 onwards. For monitoring possible methanogenic activity in the cells, the CH_4 concentration was measured from the sand layer solutions from day 127 onward.

At day 21, it was noticed that some of the solution in the sand layers

had drained out. Subsequently, the lost solution volume was replenished with AGW 24 h before each sampling for the next six sampling points (until day 125). Draining of the sand layer solutions (in total 168–226 mL; Table S1) presumably resulted from the swelling of bentonites. At days 146 and 167, sampling of the sand layer solution was performed simultaneously with re-compaction of the bentonite blocks (described in Chapter 2.2) by using the solution exiting the cells (Table S1) as a sample. At the end of the experiment (days 370–454), the cells were opened in the anaerobic hood. Samples were collected from the sand to determine the number of microorganisms, the total microbial activity and the elemental composition of precipitates. After that, samples were collected from the bentonite blocks. The surface layer (0–1 cm) was cut off from the block and the bentonite was homogenized, after which a sample was collected for enumerating the viable SRM.

2.5. Analytical methods and calculations

All the sand layer solution samples were filtered (0.45 μm) and analyzed immediately after sampling, except for the redox and pH sample (not filtered) and the sulfate and total iron samples (stored at –20 °C and 4 °C after filtration, respectively). One parallel sample of the sand layer solution was used for each analysis. The E_h (BlueLine 31 Rx; Ag/AgCl reference system) and pH (Hamilton Slim Trode; SFS 3021) were measured from open vials in the anaerobic hood (in the absence of O_2) at 21 °C. The measurement took 10–30 min due to the redox electrode slowly reaching a thermal equilibrium with the sample (Nordstrom & Wilde, 2005). Redox potentials were corrected for temperature with respect to SHE (25 °C; Sawyer et al., 1995). The concentrations of DIC (as CO_2) and dissolved CH_4 were determined by a headspace technique from an acidified sample (Trimmer et al., 2009) via gas chromatography (Kinnunen et al., 2015; Maanoja & Rintala, 2015). The concentration of DOC was measured with a total organic carbon analyzer (Shimadzu TOC-V$_{CPH}$) by manual injections (SFS-EN 1484; Stubbins & Dittmar, 2012). The concentration of dissolved sulfide was determined from an alkalified sample with an ultraviolet–visible spectrophotometer (Shimadzu UV-1700; Cord-Ruwisch, 1985). The concentration of total iron was analyzed from preserved samples (1% v/ v 67%–69% HNO_3) with inductively coupled plasma spectrometry by ALS Finland Oy. The concentration of sulfate was measured via ion chromatography (Dionex ICS-1600; SFS-EN ISO 10304). The column (IonPac AS22 4 × 250 mm), suppressor (ASRS 300, 4 mm), and detector were at 30 °C, 31 mA, and 30 °C, respectively, and the eluent was 4.5 mM Na_2CO_3/1.4 mM $NaHCO_3$ at 1.2 mL min^{-1}.

The moisture and total solids content of bentonite and sand were determined by gravimetry ($n = 2–3$; APHA, 1995). The morphology of precipitates in sand grains ($n = 23–33$) was determined by a scanning electron microscope (Jeol JSM-IT-500) at 15 or 20 kV accelerating voltages. Quantitative elemental analysis of the precipitates was determined by energy dispersive spectrometry (EDS) from sand grains mounted on carbon adhesive tape after carbon evaporation (Agar Turbo Carbon Coater). Iron and sulfur were targeted in the EDS analysis because it was assumed that the sulfide formed in the sand layers would precipitate out as mackinawite (FeS) or greigite (Fe_3S_4) having a Fe/S molar ratio of 1.0 or 0.75, respectively (Gramp et al., 2010).

The activity of the overall microorganisms in the sand and AGW samples (400 mg wet mass or 120 μL, both $n = 3$) was estimated by measuring the adenosine triphosphate (ATP) concentration with a luminometer (Plate Chameleon Multilabel Detection Platform, Hidex; Velten et al., 2007). To estimate the potential activity of the SRM in the sand, sulfate reduction rates (SRRs) were determined with a post-experiment batch assay. Subsamples of sand (10 g wet mass, $n = 4$) were incubated in 140 mL of medium described in DSMZ (2017) with the following modifications (in 1 L of AGW): 500 mg Na-lactate, 50 mg yeast extract, 50 mg K_2HPO_4, 100 mg NH_4Cl, 680 mg SO_4^{2-} from Na_2SO_4 and 55 mg $NaHCO_3$. Three parallel samples were used to

Table 2
Grain density and TS content (mean $\pm$ SD, $n = 2–3$) of the bentonites, and the characteristics of the bentonite blocks and sand layers in the experimental cells with different bentonites at different stages of the experiment.

Bentonite	Wyoming		Indian		**Bulgarian**	
Grain density (kg m^{-3})	2780[a]		2910[b]		2670[c]	
TS (ground, deoxygenated) (%$_{wm}$)	90.8 $\pm$ 0.093		88.6 $\pm$ 0.239		89.0 $\pm$ 0.010	
Object / Cell	**UNIN**	**INOC**	**UNIN**	**INOC**	**UNIN**	**INOC**
Bentonite as compacted initially						
Bentonite (kg$_{dm}$)	6.95	6.98	6.99	7.02	6.86	7.05
Liquid (kg)[d]	2.51	2.59	2.60	2.60	2.33	2.43
Volume of the block (L)	4.95	5.02	5.02	5.06	4.94	5.04
Dry density (kg m^{-3})	1405	1390	1393	1386	1388	1398
Bentonite as compacted in the beginning of the experiment (after storage of 10–108 days)						
Bentonite (kg$_{dm}$)[e]	6.95	6.97	6.99	7.01	6.85	7.05
Liquid (kg)[d]	2.57	2.65	2.66	2.65	2.55	2.49
Volume of the block (L)	5.01	5.05	5.05	5.09	4.99	5.03
Dry density (kg m^{-3})	1387	1380	1384	1379	1374	1401
Bentonite as compacted after 167 days of experiment						
Liquid (kg)[d]	2.69	2.63	2.83	2.83	2.89	2.80
Volume of the block (L)	5.12	5.15	5.18	5.22	5.22	5.15
Dry density (kg m^{-3})	1357	1353	1348	1345	1314	1368
Contents of the sand layers						
Quartz sand (kg$_{dm}$)	0.908	0.864	0.911	0.848	0.871	0.886
Liquid (L)[d,f]	0.296	0.281	0.295	0.303	0.327	0.289
Porosity (mL mL^{-1})[g]	0.47	0.47	0.47	0.49	0.50	0.47

UNIN and INOC, sand layers of the cells uninoculated or inoculated with microorganisms; TS, total solids; SD, standard deviation; SRM, sulfate-reducing microorganisms; wm, wet mass; dm, dry mass.

[a] Kiviranta & Kumpulainen, 2011.

[b] Kumpulainen & Kiviranta, 2015.

[c] Kumpulainen et al., 2016.

[d] The liquid includes the volume of artificial groundwater (11 g total dissolved solids L^{-1}, theoretical density 1006.3 kg m^{-3} at 21 °C) added to the bentonite and the ambient pore water of the bentonite.

[e] Some bentonites exited the cells during this compaction.

[f] Volume of the liquid remaining in the sand layers after installation of the plungers.

[g] Porosity was calculated using 2703 kg m^{-3} as the density of quartz sand (determined separately, data not shown).

monitor sulfate consumption by the SRM, and one sample was sterilized (60 min at 121 °C) to serve as an abiotic control sample. The samples were incubated (200 rpm) at 30 °C for 8–75 days and sampled for sulfate analysis. The SRRs were calculated from the linear part of the slope of sulfate concentration decreasing over time.

To determine the number of microorganisms, DNA was extracted from the sand and from microbial samples collected from AGW on filters (0.2 μm, Supor-200) in the beginning and at the end of the experiment. Samples (400 mg–10 g wet mass, $n = 3–5$) were stored at –20 °C before extraction (QIAGEN DNeasy PowerSoil® Kit and PowerMax Soil® Kit). The DNA extracts were analyzed for the number of SRM and total bacteria by using quantitative polymerase chain reaction (qPCR; StepOne Plus Real-Time PCR System, Applied Biosystems) to measure the number of *dsrB* (dissimilatory sulfite reductase subunit B) and bacterial 16S rRNA gene copies according to a method described in Supplementary material (Table S4). The obtained copy number of the *dsrB* gene corresponded directly to the number of SRM (Klein et al., 2001), but the number of 16S rRNA gene only gave an approximate of the number of total bacteria possessing a varying number of 16S rRNA gene copies in their genome (Stoddard et al., 2015; Větrovský & Baldrian, 2013). Thus, the number of 16S rRNA and *dsrB* gene copies measured here enabled the comparison of each microbial group size between samples but did not enable comparison of the different microbial group sizes within a sample.

The number of cultivable SRM in the compacted bentonites collected from the cells and in the uncompacted original bentonites was determined post-experiment by most probable number (MPN) technique. Serial ten-fold dilutions of an initial sample (10 g bentonite per 90 mL medium) were incubated in the medium used for the SRR batch assay at 27 °C for 28 days (Stroes-Gascoyne et al., 2010; Bengtsson & Pedersen, 2017). The bentonite samples collected from the cells were stored anaerobically in vacuum bags at 4 °C for 42–127 days (in a

descending order from Indian to Bulgarian and then to Wyoming bentonites) prior to the assay. The number of cultivable SRM in each bentonite was calculated with a statistical method based on the number of tubes showing detectable growth ($n = 4$; Koch, 1981). The molecular biological methods were not applied for the bentonite samples as they are cumbersome for the purpose and often the yield of the extracted DNA or ATP is low (Contin et al., 1995; Taborowski et al., 2019).

3. Results and discussion

3.1. Dissolution of organic matter from the bentonites

In all the cells, the concentration of DOC was 2–23 mg L^{-1} on average throughout the experiment apart from the initial peak (16–170 mg L^{-1}) observed between days 0 and 22 (Fig. 2). The initial high concentration of DOC likely resulted from mobilization of organic matter from the repeatedly compacted bentonite, and the following decrease from re-adsorption of organic matter to bentonite. Presumably, the observed DOC consisted mostly of the organic matter dissolved from the bentonites and to smaller extent organic products (e.g., volatile fatty acids) and dissolved extracellular polymeric substances released from the biofilm and microorganisms attached to the pre-enriched sand (Muyzer & Stams, 2008; Decho & Gutierrez, 2017). However, the possible amount of microbiologically produced organic matter was assumed to be insignificant relative to the amount of organic matter dissolving from the bentonites as the DOC concentrations remained generally lower in the inoculated than in the uninoculated cells during the experiment.

In the inoculated cells of Wyoming and Indian bentonites, the concentrations of DOC were lower (10 and 2 mg L^{-1} on average, respectively) than in their corresponding uninoculated cells (11 mg L^{-1} on average, and in the range of 9–53 mg L^{-1}, respectively; Fig. 2a, b).
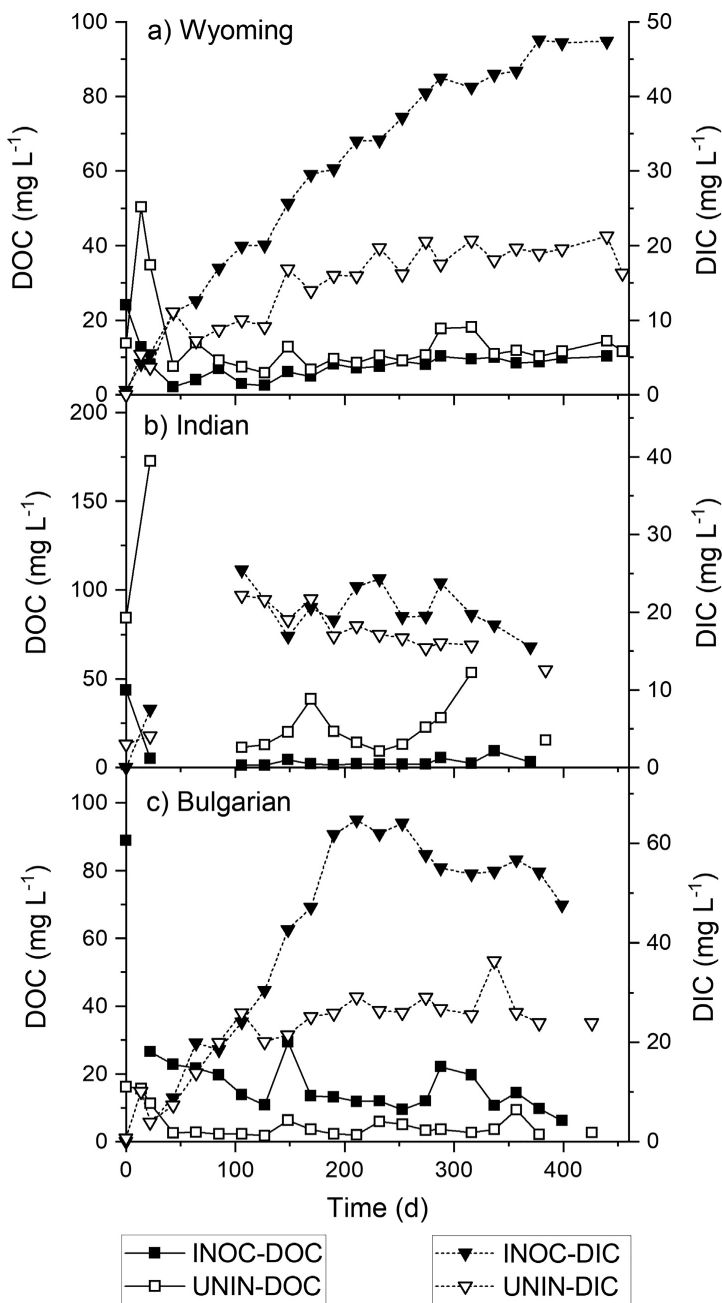
Fig. 2. Dissolved organic and inorganic carbon (DOC, DIC) in the solution of sand layers (inoculated [INOC] or uninoculated [UNIN] with microorganisms) of the experimental cells with different bentonites (a–c). Note the different scales on the y-axes.

This difference was likely resulting from microbial consumption of DOC in the inoculated cells. By contrast, in the inoculated cell of the Bulgarian bentonite, the concentration of DOC was higher than in the corresponding uninoculated cell (16 and 3.6 mg L^{-1} on average, respectively; Fig. 2c). The opposite trend compared to the cells of the other two bentonites possibly resulted from re-compaction of the swelled Bulgarian bentonite of the uninoculated cell before the experiment, when more DOC desorbed from that bentonite block (14% of the initial DOC) than from the other blocks (< 0.2%; Chapter S3, Table S3). The second re-compaction of the bentonites at days 146 and 167 induced an increase in the DOC concentrations of the uninoculated and inoculated cells of Indian and Bulgarian bentonites (by 25 and 18 mg L^{-1}, respectively; Fig. 2b, c). As a result, the microbial activity could have increased temporarily in these cells, but that was not indicated by the other measured parameters (for example no increase in concentration of DIC due to increased respiration at days 146 and 167; Fig. 2; Chapter 3.2).

When the average DOC concentrations in the cells (2–23 mg L^{-1}) were compared to the TOC "reservoir" in the bentonite blocks (1100–1500 mg TOC kg^{-1}; Table 1), it could be concluded that in case of all studied bentonites only a small part of the bentonite organic matter (0.01%–0.06% TOC w/w) was readily soluble to the water phase of the sand layer. This outcome agreed with the earlier findings of, for example, Marshall et al. (2015) who reported that only < 0.1% of TOC was water-soluble from uncompacted Wyoming bentonite. Although the fraction of water-soluble organic matter in the bentonites was low, the results indicated that organic matter became dissolved from bentonites with dry densities of 1314–1368 kg m^{-3}, which are similar to the densities potentially occurring at the interfaces of bentonite and host rock and in the backfill of a SNF repository (Autio et al., 2013). The use of higher dry density (e.g. 1780 kg m^{-3}) could result in lower dissolution of organic matter (Hallbeck, 2010).

3.2. Concentration of inorganic compounds in the sand layer solution

The concentration of DIC increased in all cells with time (until days 106–440) partly because of dissolution of calcite from the bentonites (Melamed & Pitkänen, 1996). The DIC concentrations in the inoculated cells (48, 25, and 57 mg L^{-1} on average at the highest in the cells with Wyoming, Indian, and Bulgarian bentonites, respectively) were higher than the ones observed in their corresponding uninoculated cells (18, 22, and 26 mg L^{-1}, respectively; Fig. 2) likely because of greater microbial respiration in the inoculated cells. In both cells of Wyoming and Bulgarian bentonites, the concentration of DIC remained at the highest reached concentration (Fig. 2a, c), while in both cells of the Indian bentonite, the concentration of DIC decreased from 23 mg L^{-1} to 14 mg L^{-1} on average by the end of the experiment (Fig. 2b). One possible explanation for the decrease could be precipitation of calcium carbonates, which was induced either by SRM activity (Braissant et al., 2007) or by abiotic processes occurring at high calcium concentration (1300 mg L^{-1} in AGW alone) and alkaline pH (Morse et al., 2007). The pH value of the sand layer solution was initially 6.0–6.5 in all cells, and it increased in both cells of Wyoming and Bulgarian bentonites to 8.0 and 7.5, respectively (Fig. 3a, c). In the cells of the Indian bentonite, the pH increased to a slightly lower value in the inoculated cell (7.7 on average) than in the uninoculated cell (8.0; Fig. 3b).

The concentration of sulfate increased with time in all cells due to dissolution of gypsum (Melamed & Pitkänen, 1996), and the highest concentrations were observed in the uninoculated cells (3200, 2100, and 245 mg L^{-1} with Wyoming, Indian, and Bulgarian bentonites, respectively; Fig. 4). After approximately 100 days of operation, the concentrations of sulfate started becoming increasingly lower in the inoculated than in the uninoculated cells of all bentonites (Fig. 4) indicating activation of SRM in the inoculated cells. The results suggested that the SRM could have been active also in the uninoculated cells of Wyoming and Bulgarian bentonites because the highest concentrations

of sulfate observed in these cells were not as high as could have been expected based on the sulfate contents of the bentonites (0.12, 0.06 and 0.05 wt-% SO_4-S in Wyoming, Indian and Bulgarian bentonites; Table 1).

The differences in the sulfate concentration between the inoculated and uninoculated cells in Wyoming, Indian, and Bulgarian bentonites (at highest 263, 683, and 247 mg L^{-1}) could have theoretically resulted in the production of 88, 228, and 83 mg L^{-1} of sulfide, respectively. However, the measured concentration of sulfide was low in all cells during the experiment (< 4 mg L^{-1}). The evolution of total iron concentration in the cells (Fig. 4) suggested that the formed sulfide had precipitated as iron sulfide. In the inoculated cells, the total iron concentration decreased from 1.9, 0.13, and 7.8 mg L^{-1} in Wyoming, Indian, and Bulgarian bentonites, respectively to < 0.01–0.2 mg L^{-1} around the same time when the difference in the sulfate concentration between inoculated and uninoculated cells developed (Fig. 4). The concentration of total iron was lower in the uninoculated cells than in the corresponding inoculated cells (Fig. 4), which could have resulted from the activity of microorganisms that promoted dissolution of iron from the bentonites in the inoculated cells (Colombo et al., 2013). In the uninoculated cells of Wyoming and Indian bentonites, the concentration of iron did not vary to a great extent (Fig. 4a, b). However, in the uninoculated cell of the Bulgarian bentonite, there was a period (days 127–288) when the concentration increased from 0.2 to 2.1 mg Fe L^{-1} on average (Fig. 4c). This increase could also have attributed to the activity of microorganisms (Colombo et al., 2013) as the presence of ATP in the sand layer of the uninoculated cell of the Bulgarian bentonite in the end of the experiment indicated activation of indigenous microorganisms (discussed in Chapter 3.2).

The possible precipitation of sulfide as iron sulfides was confirmed by determining the elemental composition of the precipitates in the sand after the experiment. In the inoculated cells of Wyoming and Bulgarian bentonites, the molar ratio of Fe/S in the precipitates was 0.97 and 6.5 on average, respectively (ranges of 0.1–3.4 and 0.5–22.9; Table 3), and these precipitates were identified as iron sulfides (FeS, Fe_3S_4). In the other cells, the molar ratio of Fe/S was considerably higher, 23.3 on average in the inoculated cell of the Indian bentonite and 38.8–46.4 in the uninoculated cells of all bentonites (Table 3). Thus, these precipitates were likely not FeS or Fe_3S_4, but possibly different iron oxides or hydroxides such as hematite (Fe_2O_3), magnetite (Fe_3O_4), goethite (FeOOH) or ferrous hydroxide (Fe(OH)$_2$) (Anthony et al., 2001). These findings highlight the role of bentonites as a sink of the formed sulfide, at least via dissolving iron, which immobilizes soluble sulfide as solid iron sulfides. Another possible route of immobilization of sulfide would be via diffusion of sulfide in the bentonite, where it reacts with the ferric iron of the bentonite minerals forming FeS or S^0 (Pedersen et al., 2017).

In the sand layer solution of the inoculated cells of Wyoming and Bulgarian bentonites, the E_h decreased from 200–325 mV to < 0 mV (vs. SHE) by days 150 and 110, respectively (Fig. 3a, c) and continued to decrease throughout the experiment (to –150 mV on average; Fig. 3a, c). The E_h likewise decreased in the sand layer solution of the uninoculated cells of Wyoming and Bulgarian bentonites (to 10 and 100 mV, respectively) but did not reach values as low as those in the corresponding inoculated cells. Similar evolution of the redox conditions, from oxidizing to anaerobic initially and then to increasingly reducing, is expected to occur in the repository following chemical oxidation reactions (e.g., those of Cu^0 and minerals of bentonite and host rock), microbial respiration, and reduction activity (Wolfaardt & Korber, 2012; King et al., 2017). The increasingly negative E_h values observed in the inoculated cells of the Wyoming and Bulgarian bentonites (Fig. 3a, c) were reducing enough to enable activity of SRM, which has been reported to occur below –50 mV (vs. SHE; Frindte et al., 2015). The cells with Indian bentonite deviated from the other cells in terms of evolution of E_h as in both cells of Indian bentonite, the E_h of the sand layer solution remained > 200 mV throughout the experiment
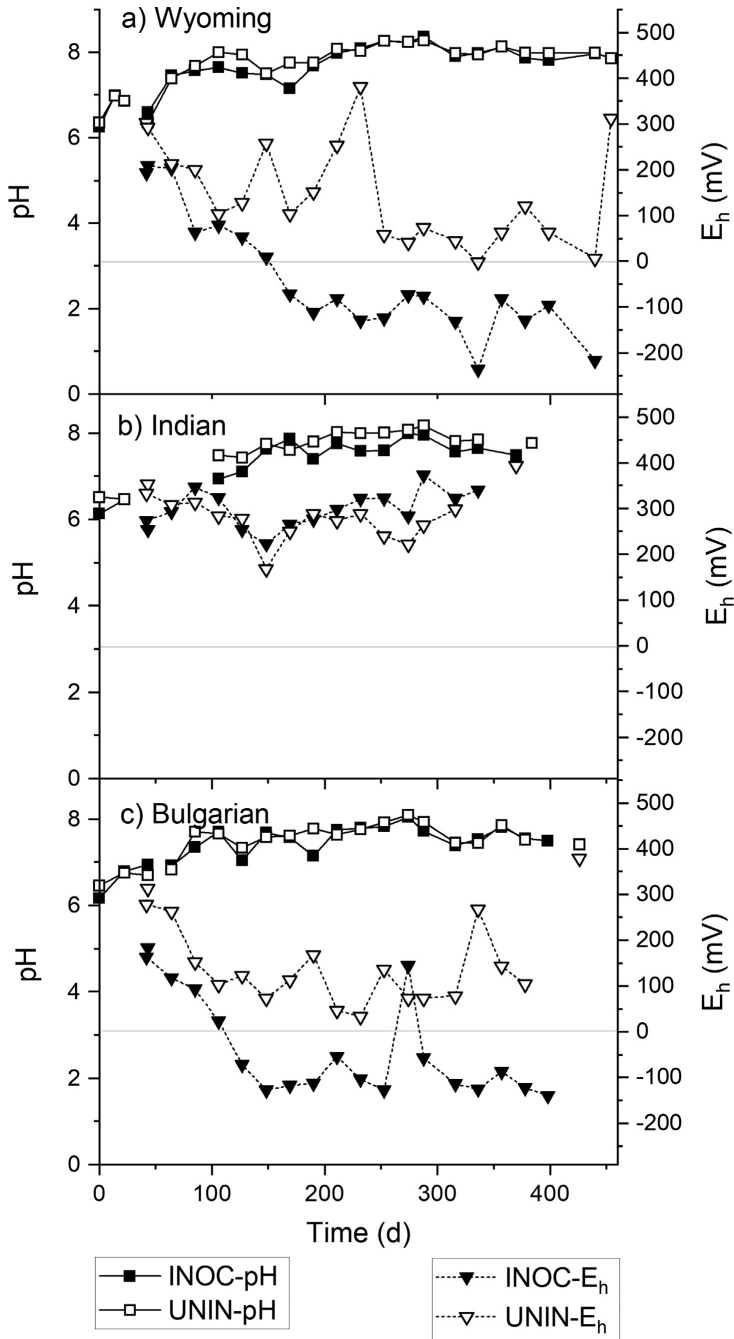
Fig. 3. Measured pH and redox potential (E_h vs. standard hydrogen electrode) of the solution in sand layers (inoculated [INOC] or uninoculated [UNIN] with microorganisms) of the experimental cells with different bentonites (a–c).

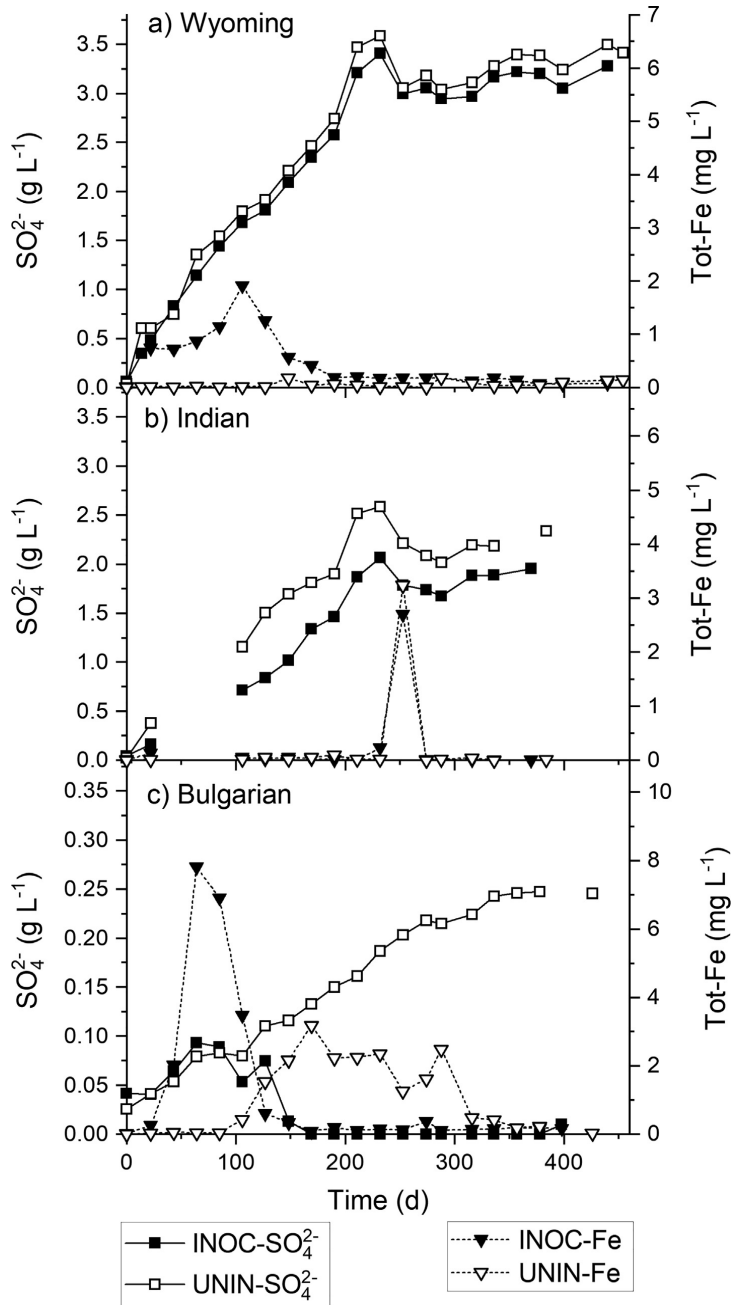**Fig. 4.** Dissolved sulfate and total iron in the solution of sand layers (inoculated [INOC] or uninoculated [UNIN] with microorganisms) of the experimental cells with different bentonites (a–c). Note the different scales on the y-axes.

Table 3

Molar ratio of Fe/S in the precipitates of the sand grains (mean [min–max], n = 24–43) and ATP concentration, SRRs, and copy numbers of *dsrB* and 16S rRNA genes in the sand and solution of the experimental cells with different bentonites (mean $\pm$ SD, n = 2–9).

Cell		Fe/S (mol mol^{-1})	ATP (nmol L^{-1})	SRR[a] (mg SO$_4{}^{2-}$ L^{-1} d^{-1})	*dsrB* of SRM (·10^5 copies L^{-1})	16S rRNA of bacteria (·10^5 copies L^{-1})
Artificial groundwater-based solution added in the cells in the beginning						
UNIN	Initial all	n.a.	< 0.09	n.m.	15.2 $\pm$ 9.90	8.5 $\pm$ 0.87
INOC	Initial all	n.a.	2.8 $\pm$ 0.08	n.m.	9501 $\pm$ 391	4019 $\pm$ 120

Cell		Fe/S (mol mol^{-1})	ATP (nmol kg^{-1})	SRR[a] (mg SO$_4{}^{2-}$ g^{-1} d^{-1})	*dsrB* of SRM (·10^5 copies g^{-1})	16S rRNA of bacteria (·10^5 copies g^{-1})
Sand added in the cells in the beginning						
UNIN	Initial all	n.m.	0.20 $\pm$ 0.037	n.m.	BLOD[b]	BLOD[b]
INOC	Initial all	n.m.	0.35 $\pm$ 0.034	n.m.	BLOD[c]	0.92 $\pm$ 0.26
Sand collected from the cells at the end						
UNIN	Wyoming	46.4 [3.4–211]	0.13 $\pm$ 0.075	0	0.79 $\pm$ 0.03	0.64 $\pm$ 0.13
	Indian	38.8 [7.6–129]	< 0.0002	0	BLOD[c]	1.5 $\pm$ 0.10
	Bulgarian	44.3 [3.8–81.3]	0.38 $\pm$ 0.098	0.94 $\pm$ 0.260	1.0 $\pm$ 0.29	BLOD[a]
Sand collected from the cells at the end						
INOC	Wyoming	0.97 [0.1–1.8]	2.0 $\pm$ 0.13	2.0 $\pm$ 0.59	8.5 $\pm$ 5.13	1.5 $\pm$ 0.03
	Indian	23.3 [1.3–139]	2.3 $\pm$ 0.27	2.1 $\pm$ 0.40	2.9 $\pm$ 2.79	5.2 $\pm$ 0.57
	Bulgarian	6.5 [0.5–22.9]	8.1 $\pm$ 0.83	0.79 $\pm$ 0.257	51.8 $\pm$ 26.0	1.8 $\pm$ 0.26

UNIN and INOC; the sand layers of the cells were <u>unin</u>oculated or <u>inoc</u>ulated with microorganisms; ATP, adenosine triphosphate; SRR, sulfate reduction rate; *dsrB*, dissimilatory sulfite reductase subunit B; rRNA, ribosomal ribonucleic acid; SRM, sulfate-reducing microorganism; SD, standard deviation; n.a., not applicable; BLOD, below limit of detection; n.m., not measured.

[a] Activity of SRM in the sand was measured post-experiment as a sulfate reduction rate in a batch assay.

[b] LOD 600 copies of 16S rRNA µL^{-1} and 600 copies of *dsrB* µL^{-1} of DNA extract.

[c] LOD 6000 copies of *dsrB* µL^{-1} of DNA extract. The LOD was determined separately for each run.

(Fig. 3b). Consequently, the growth of SRM requiring reduced conditions was highly unlikely in the sand layers of the cells with Indian bentonite. The redox potential of bentonites is affected by the presence of redox active iron species, which include both electron donating Fe^{3+} (e.g. hematite) and electron accepting Fe^{2+} minerals (e.g. siderite, pyrite) (Hofstetter et al., 2014). Consequently, the high redox potentials observed in the cells with Indian bentonite were possibly resulting from the presence of microcrystalline Fe(III) oxide phases within the comparatively large iron oxide pool of the Indian bentonite (Table 1). Due to the higher solubility of poorly crystalline iron oxide phase compared with goethite or hematite, an increased Fe^{3+}/Fe^{2+} activity ratio in solution might have developed in the experiments and, thus, increased the E_h values.

3.3. Activity and number of SRM and other microorganisms in the sand layers

Methane was detected from the sand layer solutions of all the six cells throughout the experiment, but the CH_4 concentration did not have a clear increasing or decreasing trend with time (Fig. S1). The observed CH_4 could be attributed to active methanogens, which were originating from the bentonites, and possibly from the groundwater of Olkiluoto in case of the inoculated cells. As CH_4 produced by methanogens was detected also from the cells of the Indian bentonite, it indicated that the conditions in the cells were anoxic even though they were not reducing (based on the measured E_h). Some methanogens have reported to be less sensitive to redox conditions than SRM, and they only require anaerobic, but not necessarily reducing, conditions for active growth (Frindte et al., 2015). In the inoculated cells of Wyoming and Indian bentonites, the concentration of CH_4 was lower (< 50 µg L^{-1}) than in their corresponding uninoculated cells (< 100 and < 650 µg L^{-1}, respectively; Fig. S1a, b), which could have resulted from the added SRM outcompeting the methanogens in the competition for the same organic compounds in the inoculated cells (Muyzer & Stams, 2008). In the cells of Bulgarian bentonite, however, the CH_4 concentration was higher in the inoculated than in the uninoculated cell (< 100 and < 10 µg L^{-1}; Fig. S1c), which could have been resulting from the lower availability of DOC in the uninoculated cell of Bulgarian bentonite due to re-compaction of the bentonite block before the

experiment.

At the start and at the end of the experiment, the total microbial activity was measured as a concentration of ATP. At the end of the experiment, for all bentonites, the concentration of ATP was higher in the inoculated than in the uninoculated cells (Table 3). The highest ATP concentration was observed in the inoculated cell of the Bulgarian bentonite (Table 3), which suggested that the organic matter or other growth conditions in that cell were more favorable for the mixed microbial community than in the inoculated cells of the other two bentonites. In the uninoculated cells of the Wyoming and Indian bentonites, the concentration of ATP was lower at the end than at the start of the experiment (≤ 0.13 vs. 0.20 nmol ATP kg^{-1}). In the uninoculated cell of the Bulgarian bentonite instead, the concentration of ATP was higher at the end of the experiment than in the sand initially (Table 3), which was likely resulting from propagation and activation of the indigenous bentonite microorganisms in the sand layer of this cell.

For determining the activity of SRM in the sand, the SRRs were measured in a post-experiment batch assay after the cells were opened. The SRM were active in the sands collected from all inoculated cells and from the uninoculated cell of the Bulgarian bentonite (Fig. 5; data for abiotic control samples in Fig. S2). The highest SRRs were observed in samples taken from the inoculated cells of Wyoming and Indian bentonites (Table 3). With the Bulgarian bentonite, the observed SRR was slightly higher in the uninoculated than in the corresponding inoculated cell even though the uninoculated cell showed considerably lower total microbial activity than the inoculated cell based on the ATP concentration (Table 3). This discrepancy could be explained by the fact that ATP was produced by other microorganisms than SRM in the sand of the inoculated cell of the Bulgarian bentonite as the ATP concentration represent a summed activity of the whole microbial population (Velten et al., 2007), while SRRs only the activity of SRM.

To further explore the number of different microbial groups in the sand and sand layer solutions, the number of *dsrB* gene copies for SRM and 16S rRNA gene copies for total bacterial community were quantified by qPCR. At the end of the experiment, more SRM and bacterial gene copies were found from the sand layers of the inoculated cells than of the uninoculated cells (Table 3). Some SRM and bacterial gene copies were also found from the uninoculated cells, and they were most likely originating from the microorganisms indigenous to the bentonites
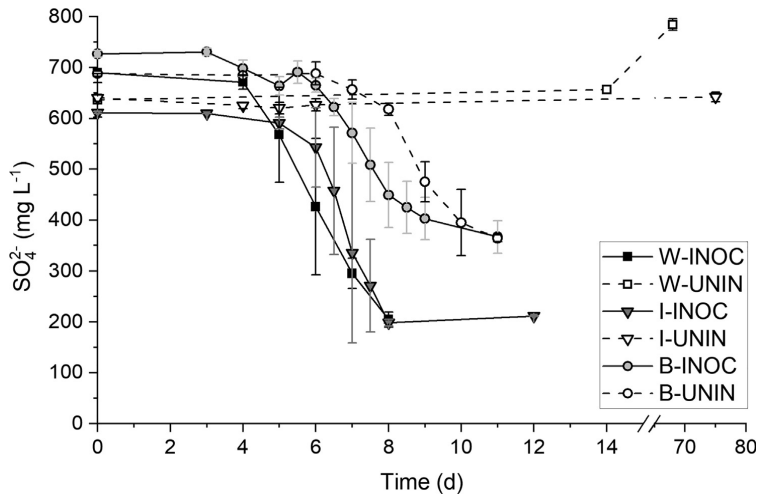
Fig. 5. Consumption of sulfate in sand inoculated (INOC) or uninoculated (UNIN) with microorganisms of the experimental cells with Wyoming (W), Indian (I) and Bulgarian (B) bentonites in a post-experiment batch assay (mean ± standard deviation, n = 2–9).

(Masurat et al., 2010; Stone et al., 2016). In the uninoculated cell of the Indian bentonite, the number of SRM gene copies remained below the limit of detection (Table 3). The number of SRM gene copies in the sand of the inoculated cells of the Indian and Bulgarian bentonites were higher and lower, respectively, than could have been expected based on the high E_h value (inhibiting the growth of SRM) and low SRR determined for these cells (Fig. 3b; Table 3). A possible reason for the observed inconsistency is that most of the SRM gene copies in the sand layers of these cells were originating from dormant cells (Burkert et al., 2019), which, in case of the inoculated cell of the Bulgarian bentonite, might not have regained their activity in the SRR batch assay.

3.4. Indications of microbial activity inside the bentonite blocks

After the experiment, the original uncompacted bentonite, which had not been in the cells, and the surface layer of the bentonite blocks (0–1 cm) were studied for the number of viable SRM using an MPN technique. The results showed the presence of viable SRM in all original uncompacted bentonites, Indian bentonite having the highest number and Wyoming bentonite the lowest number of indigenous SRM (Table 4). In the bentonite blocks of the inoculated cells of Wyoming and Indian bentonites, the number of viable SRM was higher, while in

Table 4
MPN of SRM in the compacted bentonite of the experimental cells after the experiment and in the original uncompacted bentonite.

Bentonite	Cell	MPN of SRM (g^{-1} bentonite) (lower–upper 95% confidence interval)
Wyoming	Uncompacted	370 (100–1300)
	UNIN	210 (72–620)
	INOC	660 (160–2800)
Indian	Uncompacted	7500 (1900–30000)
	UNIN	2900 (1100–7900)
	INOC	14,000 (4100–47000)
Bulgarian	Uncompacted	3800 (100–14000)
	UNIN	2200 (750–6500)
	INOC	1200 (360–3800)

MPN, most probable number; SRM, sulfate-reducing microorganisms; UNIN and INOC, sand layers of the cells were uninoculated or inoculated with microorganisms.

the bentonite block of the inoculated cell of the Bulgarian bentonite it was lower than the number of SRM in the original uncompacted bentonites (Table 4). In the bentonite blocks of all uninoculated cells, the number of SRM was lower than in the original uncompacted bentonites. It should be noted, however, that the samples were stored for 42–172 days, which could have decreased the number of viable SRM as the viability of anaerobic microorganisms has been shown to decrease with increasing storing time at 4 °C (Haavisto et al., 2019).

The MPN results suggested that some of the microbial activity, which was observed in the cells, but could not be shown to have occurred in the sand layers, had occurred inside the bentonite blocks. For example, the largest difference in the sulfate concentration was observed between the inoculated and uninoculated cells of the Indian bentonite (683 mg L^{-1}; Fig. 4b), although the E_h was not optimal for the SRM activity. Therefore, the increase in the number of SRM in the bentonite block of the inoculated cell of the Indian bentonite indicated that the active SRM were likely located within the bentonite. Given that a similar increase in the number of viable SRM was likewise observed in bentonite of the inoculated cell of the Wyoming bentonite, where the sulfate concentration of the sand layer solution remained unexpectedly low (compared to SO$_4$-S content; Chapter 3.2), the microbial activity in the Wyoming bentonite cannot be excluded either. While demonstrating the threshold densities for microbial activities in the bentonites was not the purpose of this study, the results indicate that the dry densities of the bentonites (1314–1368 kg m^{-3}) in the experiment were not high enough to fully inhibit the microbial activity. These findings agree with the threshold dry densities of sulfate-reducing activity reported for Wyoming and Indian bentonites (< 1374 kg m^{-3}; Bengtsson & Pedersen, 2017). For the Bulgarian bentonite, no threshold density for sulfate-reducing activity has been reported. Nonetheless, the present results suggested that the cultivability of the bentonite SRM (external and indigenous) slightly decreased at the dry density created in the experiment (1341 kg m^{-3} on average) compared to the number of SRM in the uncompacted bentonite. Even less microbial activity could have occurred within the compacted bentonite blocks if the dry densities had not decreased during the experiment following from the swelling occasions.

3.5. Implications

Several results evidenced that the SRM had been active in the cells during the experiment; the difference in the sulfate concentrations of the sand layer solution between the inoculated and uninoculated cells (Fig. 4), formation of sulfide-containing precipitates in the sand (Table 3), and increased SRRs and numbers of SRM gene copies in the sand of the inoculated versus uninoculated cells. Thus, the results indicate that the organic matter dissolving from all the studied bentonites can sustain microbial sulfate reduction. The SRM were either utilizing the organic matter as such or it was first degraded by other microorganisms to simpler compounds and then used by the SRM (Zavarzin et al., 2008). This experimental study therefore confirms the earlier hypothesis that SRM can use bentonite-bound organic matter as a substrate for growth, either directly or indirectly (Stone et al., 2016; Bengtsson et al., 2017). The activity of autotrophic SRM was not measured in the present experiment, but it is possible that the bentonite organic matter was first consumed by fermentative bacteria and the resulting CO_2 and H_2 by the SRM (Cassidy et al., 2015).

Several factors must be considered when evaluating the performance of the engineered barrier system. From a microbiological point of view, the Indian bentonite would be an appealing material to use in the repository because it seems to result in redox conditions that could suppress SRM activity in the EDZ. The oxidizing conditions, however, could expose the copper of the SNF canisters to corrosion and increase radionuclide mobility in case of canister failure (Posiva, 2012a, b). In case sulfide was formed in the EDZ, the Indian bentonite, which contains the highest level of ferric iron, would immobilize the sulfide by precipitating it as FeS. Out of the three studied bentonites, the Bulgarian bentonite, due to its low gypsum content, released the lowest sulfate concentration, which could result in a lower amount of sulfide produced by the SRM. However, the organic matter dissolving from the Bulgarian bentonite was found to sustain the highest overall microbial activity as measured as the highest concentration of ATP and copy number of SRM gene copies in the sand layer of the inoculated cell (Table 3). The organic matter dissolving from the Bulgarian bentonite would be beneficial for the microorganisms inhabiting the areas with lower density at the EDZ, but not necessarily for the ones inside the Bulgarian bentonite (decrease in number of SRM in the compacted bentonite blocks relative to the uncompacted bentonite; Table 4). Meanwhile, out of all the uncompacted bentonites, the Wyoming bentonite contained the lowest number of indigenous cultivable SRM, which could be considered a beneficial characteristic in terms of introducing less SRM to the repository with the bentonite.

4. Conclusions

In this study, microbial activity was studied in a unique experimental setup with microorganisms growing on a porous sand layer interconnected with compacted bentonite. Results showed that organic matter, sulfate, and iron, among other compounds, dissolved from the compacted bentonites into the sand layer solution. The microorganisms, including sulfate-reducing microorganisms (SRM), present in the sand layers grew on the organic matter and other compounds that dissolved from compacted bentonites. The SRM indigenous at least to the Bulgarian bentonite became active in the uninoculated cells during the experiment. Organic matter dissolving from all the studied bentonites (Wyoming, Indian, and Bulgarian) sustained equally the growth of microorganisms. The bentonites differed in the ions leached to the sand layer solution, which, in turn, created conditions where the SRM activity was suppressed in the cells with the Indian bentonite. These findings showed that the bentonites used to seal spent nuclear fuel repositories were able to sustain sulfate reduction in areas with lower density and immobilize possibly formed sulfides through precipitation with iron; clay mineralogy seemed to play a role in the extent of sulfate reduction and immobilization in the studied density range.

CRediT authorship contribution statement

Susanna Maanoja: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Aino-Maija Lakaniemi:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **Leena Lehtinen:** Methodology, Investigation. **Linda Salminen:** Methodology, Investigation. **Hannele Auvinen:** Conceptualization, Methodology, Investigation, Writing - review & editing. **Marika Kokko:** Conceptualization, Methodology, Formal analysis, Writing - review & editing. **Marja Palmroth:** Conceptualization, Methodology, Writing - review & editing. **Eveliina Muuri:** Resources, Writing - review & editing. **Jukka Rintala:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clay.2020.105746.

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Supplementary material for:

Compacted bentonite as a source of substrates for sulfate-reducing microorganisms in a simulated excavation-damaged zone of a spent nuclear fuel repository

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S1. Masses of bentonite and volumes of AGW required for the preparation of saturated bentonite blocks

The required mass of dry bentonite (m_s) was calculated according to Eq. S1, where ρ_d was the target dry density (1400 kg m^{-3}) and V was the predefined volume of the compacted bentonite block (5.027 L).

$$m_s = \rho_d \cdot V \qquad (S1)$$

At full saturation, the total volume of liquid in the compacted bentonite block equaled the space not taken up by the solids (i.e., the void volume). Consequently, the total volume of liquid (V_w) at the target dry density was calculated according to Eq. S2, where ρ_s was the grain density (2670–2910 kg m^{-3}; Table 2).

$$V_w = V - \frac{m_s}{\rho_s} \qquad (S2)$$

The volume of artificial groundwater (AGW) to be mixed with the bentonite (V_{wa}) was calculated according to Eq. S3, where V_{wi} was the volume of the internal water (i.e., moisture) in the bentonites.

$$V_{wa} = V_w - V_{wi} \qquad (S3)$$

The mass of internal water (m_{wi}) was calculated according to Eq. S4, where *TS* was the total solids content of the bentonites (88.6–90.8%$_{wet\_mass}$; Table 2).

$$m_{wi} = \frac{m_s}{TS/100} - m_s \qquad (S4)$$

Next, the mass of internal water was converted into volume by using Eq. S5, where ρ_{AGW} was the density of AGW (1006.3 kg m^{-3} at 21°C).

$$V_{wi} = \frac{m_{wi}}{\rho_{AGW}} \qquad (S5)$$

AGW in excess of 5% (v/v) was added to the bentonite mixture to compensate for the volume lost during mixing and other working stages; volume loss was quantified during preliminary compaction testing (data not shown). After bentonite and AGW were mixed, the moisture content of the bentonite was checked and more AGW was added if the moisture content did not match the theoretical bentonite void volume at the target density (i.e., V_w).

S2. Swelling and re-compaction of the bentonite blocks before the experiment

S2.1 Swelling during storage and re-compaction before assembling the sand layers

Bentonite blocks were compacted for the six cells one after another and, hence, some of them were stored longer (108 days) than the others (10 days) before the experiment was started at the same time with all the cells. For starting the experiment, the assemblance of the sand layers was started with the uninoculated cell of the Bulgarian bentonite by filling the sand sleeve with sand (0.871 kg$_{dry\_mass}$) and AGW (299 mL; sand layer construction described in detail in Chapter 2.3). Soon after, it was noticed that the Bulgarian bentonite had adsorbed the added AGW (65 mL on the sinter for storage and in the sand sleeve for start-up of the experiment) and swelled by 326 mL (Table S1). At this point, the unconstrained bentonites in the other five cells were also found to have swelled by 61–134 mL (Table S1) after adsorbing

the AGW added on the sinters for storage (50–65 mL). Thus, the blocks were re-compacted

to the target density before assembling the sand layers for start-up of the experiment.

Table S1. Characteristics of the bentonite blocks in the experimental cells after swelling

(during storage and after days 0–126) and re-compaction (days 146 and 167).

Bentonite Object / Cell	Wyoming		Indian		Bulgarian	
	UNIN	INOC	UNIN	INOC	UNIN	INOC
Bentonite blocks after swelling during storage of 10–108 days						
AGW adsorbed by the bentonite block in total (mL)	65	65	60	50	364[a]	60
Increase in volume of the bentonite block (mL)	90	134	61	83	326[a]	102
Dry density before re-compaction (kg m^{-3})	1380	1354	1376	1364	1302	1370
Liquid exiting the cells in re-compaction (mL)	2.3	1.4	0	0	150	0
Swelling of the bentonite blocks between days 0 and 126 of the experiment						
AGW adsorbed by the bentonite blocks in total (mL)	168	170	194	206	213	226
Increase in volume of bentonite blocks (mL)	184	190	254	272	240	328
Dry density at day 126 (kg m^{-3})	1337	1330	1317	1309	1311	1315
Re-compaction of the bentonite blocks at days 146 and 167 of the experiment						
Liquid exiting the cells in total (mL)	30	34	25	28	30	25

AGW, artificial groundwater; UNIN and INOC, sand layers of the cells uninoculated or inoculated with

microorganisms.

[a]Bulgarian bentonite of the uninoculated cell adsorbed both the AGW added on top of the sinter for storage (65

mL) and AGW added in the sand sleeve during assemblance of the sand layer for start-up of the experiment

(299 mL).

S2.2 Quantification of changes in sulfate and DOC contents of the bentonite blocks

To quantify the effect that swelling of the bentonite blocks during the storage and re-

compaction before the experiment had on the bentonites, mass balances were calculated for

adsorbed and desorbed sulfate and organic carbon (DOC). The calculations described below

were carried out assuming that the whole mass of the bentonite in the block was affected by swelling and re-compaction.

The amount of ambient sulfate in the bentonite blocks (6.86–7.05 kg dry mass; Table 2) was calculated based on the theoretical sulfate contents of the bentonites (1498, 1797 and 1595 mg SO_4^{2-} kg^{-1} in Bulgarian, Indian and Wyoming bentonites, respectively, calculated from SO_4-S contents in Table 1). In total 46–52 mg SO_4^{2-} (Table S2) was added to the bentonites when mixed with AGW during the saturation stage (20 mg SO_4^{2-} L^{-1} in AGW, 2.32–2.58 L used for each bentonite block) and it contributed only by 0.2–0.5% to the total reservoir of sulfate in the bentonite-AGW mixtures. The sum of ambient sulfate in bentonite and the amount of sulfate added within AGW equaled to the total amount of sulfate contained in the compacted bentonite blocks (10323–25126 mg SO_4^{2-}; Table S2).

For storage, the sinters in the six cells were soaked with AGW (50–65 mL) to prevent them from desaturation. After storage, the sand layer of the uninoculated cell of the Bulgarian bentonite was assembled by using 299 mL AGW (described in Chapter S2.1 and in Chapter 2.3). The unconstrained bentonite blocks adsorbed the added AGW and the sulfate within, which was 7.3 mg SO_4^{2-} for Bulgarian bentonite in the uninoculated cell and 1.0–1.3 mg SO_4^{2-} for the bentonite blocks in the other cells (Table S2). When the bentonite blocks were re-compacted, in total 150 mL of liquid filtrated out from the uninoculated cell of the Bulgarian bentonite and 0–2.3 mL from the other cells (Table S2). The concentration of sulfate was determined from the filtrate of the uninoculated cell of the Bulgarian bentonite (126 mg L^{-1}) and estimated for the filtrates of the both cells of Wyoming bentonite as the volume of the filtrate was too small for analysis (303 mg L^{-1}; see details in Table S2). Thus, the total amount of sulfate that became desorbed from the bentonite blocks in re-compaction was 18.9 mg SO_4^{2-} for Bulgarian bentonite in the uninoculated cell and 0–0.7 mg SO_4^{2-} for the bentonite blocks in the other cells (Table S2). In terms of the net balance, Bulgarian

bentonite in the uninoculated cell desorbed in total 17.6 mg SO_4^{2-}, while the bentonite blocks

in the other cells adsorbed 0.6–1.2 mg SO_4^{2-} as a result of adsorption of the added AGW and

re-compaction. These amounts were, however, very small (0.002–0.2%) when compared to

the initial total amount of sulfate in the bentonite blocks (Table S2).

The ambient DOC concentration in the bentonites was calculated based on the

theoretical total organic carbon (TOC) contents of the bentonites (1100, 1500 and 1500 mg

TOC kg^{-1} in Bulgarian, Indian and Wyoming bentonites, respectively; Table 1) and by

assuming that 0.1% of TOC was water-soluble from uncompacted bentonite (Marshall et al.,

2015). The amount of DOC adsorbed by the bentonites when mixed with AGW (containing

0.141 mg DOC L^{-1}) during the saturation stage was 0.33–0.36 mg (Table S3), which

represented 3–4% to the total reservoir of DOC in the bentonite-AGW mixtures. The total

concentration of DOC contained in the compacted bentonite blocks was 7.9–10.9 mg (Table

S3).

The amount of DOC adsorbed by the bentonite blocks during swelling was

0.051 mg DOC for Bulgarian bentonite of the uninoculated cell and 0.007–0.009 mg DOC

for the bentonite blocks of the other cells (Table S3). The concentration of DOC in the

filtrates collected during re-compaction was determined for the uninoculated cell of the

Bulgarian bentonite (7.6 mg L^{-1}) and estimated for the filtrates of the both cells of Wyoming

bentonite (10.4 mg L^{-1}; Table S3). Thus, the total amount of DOC that became desorbed from

the bentonite blocks in re-compaction was in total 1.14 mg for Bulgarian bentonite of the

uninoculated cell and 0–0.024 mg for the bentonite block of the other cells (Table S3). As for

the net balance, Bulgarian bentonite in the uninoculated cell and Wyoming bentonite in both

cells desorbed in total 1.09 mg and 0.005–0.015 mg DOC, respectively, while Indian

bentonite in both cells adsorbed 0.008 mg DOC (Table S3). When compared to the initial

total amount of DOC in the bentonite blocks, the amounts sorbed by Wyoming or Indian

bentonites in all cells or Bulgarian bentonite in the inoculated cell were small (0.05–0.1%;
Table S3). For Bulgarian bentonite in the uninoculated cell, the amount of desorbed DOC
during re-compaction was slightly higher than for the others (14% of the initial DOC; Table
S3).

Table S2. Theoretical amount of sulfate in the bentonite blocks of the experimental cells
initially, after swelling during storage and after re-compaction before start-up of the
experiment.

Bentonite	Wyoming		Indian		Bulgarian	
Cell	UNIN	INOC	UNIN	INOC	UNIN	INOC
Compacted bentonites before storage						
Total SO_4 in bentonite blocks (mg)	25035	25126	12613	12660	10323	10603
> Ambient SO_4 in bentonites (mg)	24986	25074	12561	12608	10277	10555
> SO_4 added with AGW in saturation (mg)	50	52	52	52	46	48
Swelling during storage and re-compaction before start-up of the experiment						
SO_4 adsorbed by bentonite in total (mg)	1.3	1.3	1.2	1.0	7.3	1.2
> SO_4 from AGW added on sinter (mg)	1.3	1.3	1.2	1.0	1.3	1.2
> SO_4 from AGW added in sand layer (mg)	n.a.	n.a.	n.a.	n.a.	6.0	n.a.
SO_4 desorbed in re-compaction (mg)	0.7	0.4	0	0	18.9	0
> Liquid filtrated out from the cell (mL)	2.3	1.4	0	0	150	0
> SO_4 in the filtrated liquid (mg L^{-1})	303[a]	303[a]	n.a.	n.a.	126	n.a.
SO_4 net mass balance (mg)[b]	+0.6	+0.9	+1.2	+1.0	-17.6	+1.2
SO_4 net mass balance (% of initial)	0.002	0.003	0.01	0.008	0.17	0.011

UNIN and INOC, sand layers of the cells uninoculated or inoculated with microorganisms; AGW, artificial
groundwater; n.a., not applicable.

[a]The concentration of sulfate could not be measured from the liquid filtrating out from the Wyoming bentonite
blocks because the volume was too low for the analysis. Thus, the concentration was estimated based on the
concentration of sulfate in the filtrate of the uninoculated cell of Bulgarian bentonite (126 mg L^{-1}); the
concentration in the filtrate from the Wyoming blocks was assumed to be proportional to the ambient sulfate
content in the bentonite, which was 1498 mg SO_4^{2-} kg^{-1} for the Bulgarian bentonite and 1595 mg SO_4^{2-} kg^{-1} for
the Wyoming bentonite.

[b]The plus sign indicates that the bentonites retained some of the sulfate adsorbed from AGW after re-
compaction and minus sign indicates that the bentonites lost more sulfate in re-compaction that adsorbed from
the AGW.

Table S3. Theoretical amount of DOC in the bentonite blocks of the experimental cells initially, after swelling during storage and after re-compaction before start-up of the experiment.

Bentonite	Wyoming		Indian		Bulgarian	
Cell	UNIN	INOC	UNIN	INOC	UNIN	INOC
Compacted bentonites before storage						
Total DOC in bentonite blocks (mg)	10.8	10.8	10.8	10.9	7.9	8.1
> Ambient DOC in bentonites (mg)	10.4	10.5	10.5	10.5	7.5	7.8
> DOC added with AGW in saturation (mg)	0.35	0.36	0.36	0.36	0.33	0.34
Swelling during storage and re-compaction before start-up of the experiment						
DOC adsorbed by bentonite in total (mg)	0.009	0.009	0.008	0.007	0.051	0.008
> DOC from AGW added on sinter (mg)	0.009	0.009	0.008	0.007	0.009	0.008
> DOC from AGW added in sand layer (mg)	n.a.	n.a.	n.a.	n.a.	0.042	n.a.
DOC desorbed in re-compaction (mg)	0.024	0.015	0	0	1.14	0
> Liquid filtrated out from the cell (mL)	2.3	1.4	0	0	150	0
> DOC in the filtrated liquid (mg L^{-1})	10.4[a]	10.4[a]	n.a.	n.a.	7.6	n.a.
DOC net mass balance (mg)[b]	-0.015	-0.005	+0.008	+0.007	-1.09	+0.008
DOC net mass balance (% of initial)	0.14	0.05	0.08	0.06	13.9	0.10

DOC, dissolved organic carbon; UNIN and INOC, sand layers of the cells uninoculated or inoculated with microorganisms; AGW, artificial groundwater; n.a., not applicable.

[a]The concentration of DOC could not be measured from the liquid filtrating out from the Wyoming bentonite cells because the volume was too low for the analysis. Thus, the concentration was estimated based on the concentration of DOC in the filtrate of the uninoculated cell of Bulgarian bentonite (7.6 mg L^{-1}); the concentration in the filtrate from the Wyoming blocks was assumed to be proportional to the ambient concentration of water-soluble organic carbon in the bentonites, which was 1.1 mg DOC kg^{-1} for the Bulgarian bentonite and 1.5 mg DOC kg^{-1} for the Wyoming bentonite.

[b]The plus sign indicates that the bentonites retained some of the DOC adsorbed from AGW after re-compaction and minus sign indicates that the bentonites lost more DOC in re-compaction that adsorbed from the AGW.

S3. Amending the sand and AGW with microorganisms for the inoculated cells

S3.1 Pre-enrichment of sand with groundwater microorganisms

Prior to the cell experiment, the sand for inoculated cells was pre-enriched with microorganisms originating from Olkiluoto groundwater. The aim was to promote the

formation of an adhered microbial community (i.e., biofilm) that would acclimatize to the cell conditions (e.g., salinity).

In the pre-enrichment culture, organic matter leached from the Wyoming, Indian, and Bulgarian bentonites was used as a substrate for the microorganisms. Bentonite leachates were prepared by incubating bentonite with sterile and anaerobic AGW at a liquid-to-solid ratio of 40 L kg^{-1} for ≥7 days at 150 rpm. After incubation, the AGW-based leachates were separated from the bentonites by centrifugation (10 min at $10000 \times g$) and pooled (300–600 mL from each bentonite).

The pre-enrichment culture was prepared by mixing Olkiluoto groundwater (1400 mL; drill hole ONK-PVA06, collected in October 2017), a batch of carbon-free sand and the bentonite leachates. To increase the number of microorganisms in the culture, an additional batch of groundwater microorganisms was isolated from groundwater by filtering (1000 mL; 0.20 µm, Supor-200) and added to the medium by shaking the filter vigorously in the medium with tweezers. The pre-enrichment culture was incubated at $20 \pm 2°C$ for 11 weeks.

After 11 weeks of incubation, the medium of the culture was refreshed. Approximately 75% (v/v) of the old medium was removed, and fresh anaerobic bentonite leachates (500 mL each of Wyoming, Indian, and Bulgarian; produced as earlier) were added to the remaining medium. A new batch of groundwater microorganisms collected on a filter from Olkiuoto groundwater (3600 mL; ONK-PVA06, collected in January 2018) was distributed to the medium as described above. The loss of some pelagic microorganisms along the removed batch of the old medium was assumed. Thus, the old medium was filtered (0.2 µm, Supor-200), and the biomass captured on the filter was redistributed to the new medium. The pre-enrichment culture was incubated in the refreshed medium for six weeks at $20 \pm 2°C$ prior to the start of the cell experiment.

At the same time, when the medium was refreshed on week 11 of incubation, the sand was studied for the presence of sulfate-reducing microorganisms (SRM) in a batch assay to confirm that the pre-enrichment was successful. Samples of sand (10 g_{wet_mass}, $n = 6$) were incubated in two different media (8.4 g NaCl L^{-1}; Zamora & Malaver, 2012; DSMZ, 2017) for six weeks at 28°C. The medium in all the bottles turned black due to the formation of FeS, indicating that cultivable SRM were present in the pre-enrichment culture (Krieg, 1981).

When the sand layers of the inoculated experimental cells were set up in the beginning of the experiment, the sand from the pre-enrichment culture (4.7 kg) was separated from the pre-enrichment medium, which was saved for collecting the pelagic microorganisms at later stage (described in Chapter S3.2). The sand from the batch assay (60 g in total) was combined with the sand pre-enriched with groundwater microorganisms. Next, the combined sand batch was rinsed with sterile AGW to remove traces of media and the FeS precipitates formed during the batch assay. Then, the sand was divided in three sub-batches and inserted into the inoculated cells.

S3.2 Amendment of AGW with different microorganisms

The AGW used for saturating the sand layers of the inoculated cells was first amended with microorganisms from three sources:

1) Pure cultures of SRM: *Desulfobacula phenolica*, *Desulfobulbus mediterraneus*, *Desulfobulbus rhabdoformis*, *Pseudodesulfovibrio aespoeensis*, and *Desulfotomaculum acetoxidans* were obtained from DSMZ GmbH (IDs 3384, 13871, 8777, 10631 and 771, respectively). Pure cultures of the SRM were used in the experiment to create an abundant initial SRM community in the sand layers of the inoculated cells. The SRM were delivered from DSMZ GmbH in 10–15 mL of

specific media, from where the microbial cells were harvested without pre-culturing as described below.

2) Microorganisms of fresh Olkiluoto groundwater (3.6 L, ONK-PVA06, extracted in January 2018 and stored in gas-tight bottles at 4°C for three weeks) were used in the experiment to include a mixed community of native groundwater microorganisms in the sand layers.

3) Pelagic microorganisms of the pre-enrichment medium used to culture the sand (2.3 L; described in Chapter S3.1).

The pure cultures of SRM and microorganisms from groundwater and pre-enrichment medium were collected on sterile filters (0.2 μm, Supor-200). Residues of the specific media of the SRM pure cultures, pre-enrichment medium and groundwater were washed off from the filters with sterile AGW by filtering. Microorganisms captured on the filters were distributed in the batch of AGW to be added to the sand layers by shaking the filters vigorously in the solution with tweezers. Then, the AGW was divided in three sub-batches and inserted into the inoculated cells.

Table S4. qPCR thermal cycling protocols for amplification of *dsrB* and 16S rRNA genes.

Step	*dsrB*	16S rRNA
Primer pair	DSRp2060F/DSR4R[a]	Eub338F/Eub518R[b]
Initial denaturation	10 min at 95°C	10 min at 95°C
Denaturation, annealing and extension	15 s at 95°C, 30 s at 55°C, 30 s at 72°C, repeated 45×	15 s at 95°C, 60 s at 62°C, repeated 35×

qPCR, quantitative polymerase chain reaction; *dsrB*, dissimilatory sulfite reductase subunit B; 16S rRNA, 16S ribosomal ribonucleic acid.
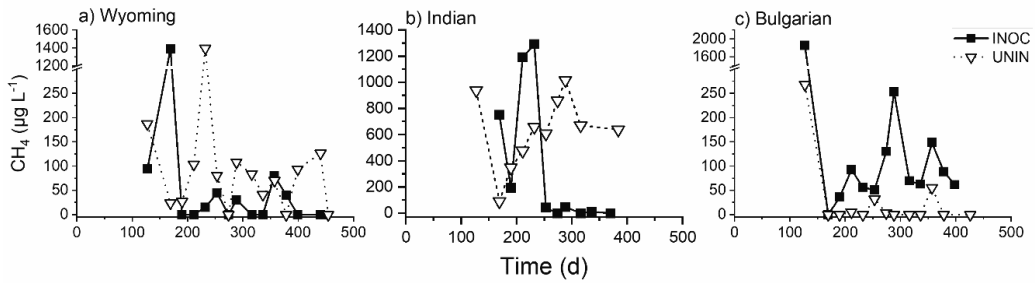
[a]Geets et al., 2006

[b]Fierer et al., 2005

Figure S1. Concentration of dissolved methane (from day 127 onwards) of the solution in the sand layers (inoculated [INOC] or uninoculated [UNIN] with microorganisms) of the experimental cells with different bentonites (a–c).
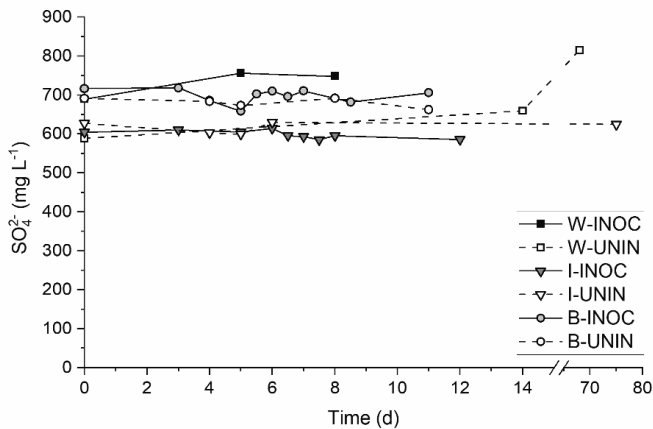

Figure S2. Concentration of sulfate in abiotic control samples of a post-experiment sulfate reduction rate batch assay (sand inoculated [INOC] or uninoculated [UNIN] with microorganisms of the experimental cells with Wyoming [W], Indian [I] and Bulgarian [B] bentonites [$n = 1$]).

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PUBLICATION
IV

The effect of compaction and microbial activity on the quantity and release rate of water-soluble organic matter from bentonites

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