HZDR-103

JOINT PROJECT: UMWANDLUNGSMECHANISMEN IN BENTONITBARRIEREN

SUBPROJECT B: EINFLUSS VON MIKROBIELLEN PROZESSEN AUF DIE BENTONITUMWANDLUNG

Nicole Matschiavelli, Jennifer Drozdowski, Sindy Kluge,
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Final Report

BMWi Project No.: 02 E 11344B

Joint project: Umwandlungsmechanismen in Bentonitbarrieren

Subproject B: Einfluss von mikrobiellen Prozessen auf die Bentonitumwandlung

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Vorhaben

Verbundprojekt UMB: Umwandlungsmechanismen in Bentonitbarrieren (UMB)

Teilprojekt B: Einfluss von mikrobiellen Prozessen auf die Bentonitumwandlung

Laufzeit des Vorhabens: 01.04.2015 bis 31.12.2018

Projektleiter: Dr. Thuro Arnold

Helmholtz-Zentrum Dresden-Rossendorf e.V.

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BMWi Förderkennzeichen: 02E11344B

I. Schlussbericht

1. Aufgabenstellung (Problem – Aufgabe – Ziel)

Ziel der Arbeiten ist die Entwicklung abgesicherter, objektiver Kriterien zur Auswahl geeigneter Bentonite für den Einsatz in Endlagern für wärmeentwickelnde Abfälle in Tonformationen. Durch ein aufeinander abgestimmtes experimentelles und modelltheoretisches Arbeitsprogramm sollen Lücken im Prozessverständnis der Bentonitumwandlung im Kontakt zu Formationslösungen geschlossen werden. Das Vorhaben „UMB“ sollte in vernetzten Einzelprojekten offene Fragen zur Bentonitstabilität klären.

Im GRS-Vorhaben 02E10538 „Fe-Bentonit“ wurden Umwandlungsmechanismen von Bentoniten im Kontakt mit unterschiedlichen Lösungen (NaCl-Lösung, Opalinuston-Lösung, Äspö-Lösung) bei Temperaturen von 25 °C, 60 °C und 90 °C untersucht. In Zusammenarbeit mit der Universität Greifswald konnte gezeigt werden, dass unterschiedliche Bentonite unter gleichen Bedingungen (gleiche Kompaktion, Kontakt mit den gleichen Lösungen und gleiche Temperatur) sich unterschiedlich verhalten /HER 06/, /HER 08/, /HER 10/, /HER 11/, /HER 12/, /HER 14/, NGU 11/, /NGU 14/, /KAS 12/.

Als Erklärung dafür wurde die unterschiedliche Puffer- / Aufnahmekapazität von gelöstem Si identifiziert /HER 14/, /NGU 14/. Es wurde eine Theorie aufgestellt, nach der der Verbleib bzw. die Abfuhr von gelöstem Silizium aus dem System Bentonit-Lösung auf die chemische Ausgangszusammensetzung der quellfähigen Montmorillonitanteile im Bentonit zurückzuführen ist. Smektite mit weniger als vier Si-Atomen pro Formeleinheit können demnach gelöstes Si aus der Lösung solange aufnehmen, bis sie die Idealzusammensetzung von vier Si-Atomen erreicht haben. Sobald diese Kapazität erschöpft ist, wird überschüssiges Si aus der Lösung ausgefällt. Dadurch werden die Smektitaggregate verklebt und ihre Quellfähigkeit reduziert, was zu einer erhöhten Permeabilität führt. Diesen postulierten Mechanismus gilt es durch die Untersuchung weiterer Bentonite abzusichern und für die chemisch-mineralogische Untersuchung ein standardisiertes Verfahren bereitzustellen /HER 12/, /HER 14/, /NGU 11/, /NGU 14/, /KAS 12/.

Eine Unsicherheit liefert in diesem Zusammenhang die Mikrobiologie. Reaktionen, die abiotisch nicht stattfinden, sind im Kontakt mit Mikroben möglicherweise als relevant anzusehen, da Mikroben spezielle Reaktionen katalysieren können. Weitgehend unbekannt ist, in welchem Umfang Umwandlungen quellfähiger Montmorillonite im Bentonit durch bakterielle Aktivität beeinflusst werden. Hierzu wurden Untersuchung des Einflusses mikrobieller Effekte auf die Alteration von

Bentoniten durch Reduktion von Fe(III) zu Fe(II) und Bildung von Sulfiden bei 25 °C, 60 °C und 90 °C (Experimente GRS, Mikrobiologische Untersuchungen HZDR) durchgeführt.

Das Ziel dieses auf den mikrobiologischen Einfluss konzentrierten Teilprojekts ist die Untersuchungen zum Einfluss von Bakterien auf die Bentonitumwandlung.

2. Voraussetzungen, unter denen das Vorhaben durchgeführt wurde

Das Institut für Ressourcenökologie des HZDR weist viele Erfahrungen in der Untersuchung der Transportprozesse von Radionukliden in der Umwelt, in natürlichen Gewässern, im nahen und entfernteren Bereich ehemaliger Uranbergbau-Stätten (Bergwerke, Halden, Abräume) und in zukünftigen Lagern für radioaktive Abfälle auf. Zum Sorptionsverhalten von Radionukliden, insbesondere von Uran, auf Mineral- und Gesteinsoberflächen sind bereits zahlreiche wissenschaftliche Publikationen entstanden.

Seit einigen Jahren beschäftigt sich das Institut für Ressourcenökologie darüber hinaus mit dem Einfluss von Mikroorganismen auf das Migrationsverhalten von Radionukliden. In der Biosphäre sind vor allem Mikroorganismen an den Stoffkreisläufen der Elemente beteiligt und führen zur Mobilisierung oder Immobilisierung vieler radioaktiver Elemente. So bestimmt die Wechselwirkung von Mikroorganismen das Migrationsverhalten von Radionuklide in der Natur und letztlich wie sehr die Radionuklide eine Bedrohung für die menschliche Gesundheit darstellen.

Das Institut verfügt über eine sehr gute Infrastruktur in radiochemischen und biologischen (Gentechnik S1) Laboren innerhalb von Kontrollbereichen mit einem umfassenden Methodenspektrum zur Aufklärung der mikrobiellen Diversität und der Speziation von Radionukliden.

3. Planung und Ablauf des Vorhabens

Das Projekt wurde in der Zeit vom 01.04.2015 bis zum 31.12.2018 durchgeführt. Aufgrund der deutlich späteren Probenentnahme von den Ansätzen der GRS war die Zielerreichung nicht möglich. Aus diesem Grund wurde im Juli 2017 ein Verlängerungsantrag bis Ende 2018 gestellt. Diesem wurde im Dezember 2017 zugestimmt. Damit war die Zielerreichung wieder möglich.

4. Wissenschaftlicher und technischer Stand, an den angeknüpft wurde

Im Gegensatz zum Einfluss anorganischer Immobilisierungsprozesse auf das Ausbreitungsverhalten von Radionukliden in der Umwelt, ist der Einfluss von

Mikroorganismen auf den Transport von Radionukliden und deren Immobilisierung/Mobilisierung bislang nur wenig erforscht. Sulfat-reduzierende Bakterien und Methan produzierende Archaeen, die in Bentoniten und Tongesteinen indigen sind, können diese Reaktion in Gang bringen und halten /MEL 11/.

Weitgehend unbekannt ist, in welchem Umfang solche Umwandlungen auf molekularer Ebene durch bakterielle Aktivität beeinflusst werden.

Bisherige Arbeiten zu mikrobiellen Effekten in Bentoniten haben sich hauptsächlich mit dem Nachweis des Vorhandenseins von aktiven Mikroorganismen in hoch kompaktierten Bentoniten und der Produktion des korrosionsfördernden Sulfids, jedoch nicht mit Bentonit-Umwandlung auseinandergesetzt /CHI 08/, /MAS 10a/, /MAS 10b/, /LYD 10/. Umfangreiche Laboruntersuchungen mit Tonmineralen und in Nährmedien gezüchteten Kulturen von anaeroben Mikroorganismen – die mit den Bentonit-eigenen Mikroorganismen durchaus identisch sein können – zeigen, dass bis zu 30 % des Fe(III) in der Smektit-Struktur innerhalb von wenigen Wochen mikrobiell – und nur 1 - 2 % abiotisch in den sterilen Kontrollen – reduziert werden können /LIU 11/, /MEL 11/, /LIU 12/, /ZHA 12/. Diese Reduktion beeinflusst die Smektit-Struktur und kann in einer Smektit-Auflösung und Mineralneubildung resultieren. Sowohl Fe(III)-reduzierende als auch Sulfat-reduzierende Bakterien und Methan produzierende Archaeen, die in Bentoniten und Tongesteinen indigen sind, können diese Reaktion in Gang bringen und halten /MEL 11/.

Das Institut für Ressourcenökologie verfügt seit einigen Jahren über Erfahrungen zur Wechselwirkung von Radionukliden und Lanthaniden auf molekularer Ebene vor allem mit Mikroorganismen. In einigen geförderten Projekten u.a. des BMBF und in EU-Projekten hat das HZDR bereits gemeinsam mit nationalen und internationalen Partnern interdisziplinär zusammengearbeitet und hierbei die mikrobiellen Einflüsse auf die Migration von Radionukliden untersucht.

5. Zusammenarbeit mit anderen Stellen

Zusammenarbeit gab es mit den Kooperationspartner (Förderkennzeichen 02E11344) von der Gesellschaft für Anlagen- und Reaktorsicherheit (GRS), Abteilung Sicherheitsanalysen, Bereich Endlagersicherheitsforschung, Braunschweig und der Ernst-Moritz-Arndt-Universität Greifswald, Institut für Geographie und Geologie, Greifswald. Das HZDR hatte eine beratende Rolle bei der Konzeption der mikrobiologischen Experimente und führte die sterile Probennahme beim Projektpartner in Braunschweig durch. Mit dem Projektpartner aus Greifswald wurde die mineralogische Charakterisierung der Bentonit Proben mittels XRD und REM/EDS realisiert.

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/HER 06/ Herbert, H.-J und Xie, M (2009): Bericht über die experimentelle Vorgehensweise im Vorhaben „Fe-Bentonit“ auf dem EBS Task Workshop, 24-26.05.2006 in Rosersberg, Sweden

/HER 08/ Herbert, H.-J.; Kasbohm, J.; Sprenger, H.; Reichelt, Chr.; Fernandez, A. M. (2008): Swelling pressures of MX-80 bentonite in solutions of different ionic strength – Clays in Natural Engineered Barriers for Radioactive Waste Confinement. Physics and Chemistry of the Earth, Parts A/B/C, 3(1), 327–342

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/NGU 11/ Nguyen-Thanh, L., Hoang-Minh, T., Herbert, H.J., Kasbohm, J (2011): Short-term experiments testing difference kinetics of Fe-rich bentonites. Euroclay 2011, Antalya, 26.06.-01.07.2011

/NGU 14/ Nguyen, T. L.; Kasbohm, J.; Herbert, H.-J.; Hoang-Minh T. (2014): "Rate of Alteration" – experiments on a series of bentonites. Presentation to be held on: International Conference on the Performance of Engineered Barriers: Backfill, Plugs & Seals – Physical and Chemical Properties, Behaviour & Evolution – organised by the 7th Framework EURATOM project PEBS; to be held at the BGR Hannover, Germany on February 6-7, 2014

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II. Eingehende Darstellung

1. der Verwendung der Zuwendung und des erzielten Ergebnisses im Einzelnen

Die Zuwendung (Personal-, Material-, Reisekosten) wurde vollumfänglich für die Durchführung der Projekte verwendet.

2. der wichtigsten Positionen des zahlenmäßigen Nachweises

Frau Dr. Nicole Matschiavelli wurde vom 01.01.2016 bis zum 31.12.2017 mit einer 50 %-igen Stelle auf dem Projekt angestellt und vom 01.01.2018 bis zum 31.12.2018 mit einer 100 %-igen Stelle als wissenschaftliche Mitarbeiterin auf dem Projekt angestellt.

Frau Sindy Kluge wurde vom 01.05.2016 bis zum 30.10.2017 (18 Monate) mit einer 100%igen Stelle als technische Mitarbeiterin auf dem Projekt angestellt.

Der Verwendungsnachweis zusammen mit den Jahresabrechnungen für die Projektlaufzeit wurde dem Projektträger gesondert zugesendet.

Reisekosten wurden für die Teilnahme an den halbjährlichen Projekttreffen bzw. Workshops des Verbundes, für Gastaufenthalte an anderen wissenschaftlichen Einrichtungen und zum Besuch von nationalen und internationalen Konferenzen aufgewendet.

3. der Notwendigkeit und Angemessenheit der geleisteten Arbeit

Da der Umfang mikrobiell beeinflusster Umwandlungen von quellfähigem Montmorillonit zu nicht quellfähigen Tonmineralen nicht bekannt ist, wurde die hier in dem Projekt beschriebenen Untersuchungen durchgeführt. Dies diente auch der Ermittlung von objektive Kriterien zur Auswahl geeigneter Bentonite für den Einsatz in Endlagern für wärmeentwickelnde Abfälle in Tonformationen zu erhalten.

4. des voraussichtlichen Nutzens, insbesondere der Verwertbarkeit der Ergebnisse im Sinne des fortgeschriebenen Verwertungsplans

Ein unmittelbarer wirtschaftlicher Nutzen ist mit den zu erzielenden Ergebnissen nicht verbunden. Der Nutzen der Ergebnisse liegt primär in ihrer Anwendbarkeit bei der Durchführung von Analysen für den Nachweis einer ausreichenden Langzeitstabilität und Dichtwirkung von Bentoniten in Tonformationen. Die Ergebnisse können von allen Organisationen, die im Auftrag des Bundes oder der Länder für die Planung und/oder

Errichtung bzw. Genehmigung eines Endlagers verantwortlich sind, angewendet werden. Die Ergebnisse sind auch für die Bewertung von Bentonitdichtungen geeignet, die im Bereich von Übertage- und Untertagedeponien zum Einsatz kommen.

Die Kenntnis potentiell relevanter Umwandlungsmechanismen auf mikrostruktureller Ebene ermöglicht die kontrollierte Konditionierung von Bentoniten im Hinblick auf eine effektive Barrierewirkung. Modelle, die das Langzeitverhalten von Bentonitdichtungen beschreiben, können auf der Grundlage der Messdaten in ihren konstitutiven Beziehungen der Thermodynamik und der Kinetik weiterentwickelt werden und tragen damit zu einem verbesserten Systemverständnis über die Wirksamkeit von Bentonitdichtungen bei. Die wissenschaftlichen und technischen Erfolgsaussichten dieses Vorhabens sind insgesamt als gut einzustufen.

Die Ergebnisse liefern außerdem einen Beitrag zu aktuellen Diskussionen über den Einfluss mikrobieller Prozesse auf die Eisen-Korrosion und die Entwicklung und die Mobilität von Gasen in der Umgebung der Bentonitdichtungen.

5. des während der Durchführung des Vorhabens dem ZE bekannt gewordenen Fortschritts auf dem Gebiet des Vorhabens bei anderen Stellen

Im Vorhabenszeitraum wurde keine relevante Literatur zum konkreten Forschungsthema publiziert.

6. Veröffentlichungen der Ergebnisse

Die Ergebnisse der vorliegenden Arbeit wurden bereits, bzw. werden noch in internationalen

Fachzeitschriften veröffentlicht. Des Weiteren wurden die Ergebnisse bei nationalen und internationalen Kongressen vorgestellt und diskutiert.

Publikationen

- Poster: „*Microbial transformation of bentonite*“ ; N. Matschiavelli, J. Steglich, S. Kluge, A. Cherkouk; 16th International Clay Conference - Clays, from the oceans to space (17. - 21.07.2017 in Granada, Spain
- Poster: „*Microbial transformation of bentonite*“ ; N. Matschiavelli, J. Steglich, S. Kluge, A. Cherkouk; GDCh-Wissenschaftsforum Chemie 2017 Interdisziplinäre Symposien Jahrestagung Nuklearchemie, 10 - 14.09.2017, Berlin, Germany
- Poster: “Bentonite – geotechnical barrier and source for microbial life” N. Matschiavelli, J. Steglich, S. Kluge, and A. Cherkouk; VAAM-DGHM 2017, Würzburg, Germany

- Report: “Bentonite – geotechnical barrier and source for microbial life**”** N. Matschiavelli, J. Steglich, S. Kluge, and A. Cherkouk; Annual report 2016; Institute of Resource Ecology, page 57
- Report: „Evolution of microbial diversity in bentonite-microcosms“; N. Matschiavelli, S. Kluge, V. Prause, A. Meleshyn and A. Cherkouk; Annual report 2017; Institute of Resource Ecology, page 54
- Oral presentation: “Microbial Influence on Bentonite Transformation”; N. Matschiavelli, J. Drozdowski, S. Kluge, T. Arnold and A. Cherkouk, UMB annual project meeting; GRS Braunschweig, 02.06.2016
- Oral presentation: “Microbial Influence on Bentonite Transformation”; N. Matschiavelli, J. Drozdowski, S. Kluge, T. Arnold and A. Cherkouk, UMB annual project meeting; GRS Braunschweig, 22.09.2017
- Oral presentation: “Microbial Influence on Bentonite Transformation”; N. Matschiavelli, J. Drozdowski, S. Kluge, T. Arnold and A. Cherkouk, UMB annual project meeting; GRS Braunschweig, 24.05.2018
- Oral presentation: “Microbial Aspects on Bentonite Transformation”; N. Matschiavelli, J. Drozdowski, S. Kluge, T. Arnold and A. Cherkouk, UMB final project meeting; GRS Braunschweig, 13.12.2018

III. Erfolgskontrollbericht

1. Beitrag des Ergebnisses zu den förderpolitischen Zielen des Förderprogamms

Das Bundesministerium für Wirtschaft und Technologie (BMWi) fördert anwendungsbezogene Grundlagenforschung als Vorsorgemaßnahme für die zukünftige Sicherheit von Mensch und Umwelt. Das zugehörige Förderkonzept 2011 – 2014 ist eine Aktualisierung und partielle Fortschreibung der früheren Förderkonzepte von BMWi und dem Projektträger Karlsruhe - Wassertechnologie und Entsorgung (PTKA-WTE). Die Thematik schließt die Bearbeitung grundlegender Sicherheitsfragen zur Endlagerung radioaktiver Abfälle in Endlagern in Salz- und Tonformationen ein.

Gefördert wird die Vertiefung von Kenntnissen über sicherheitsrelevante Prozesse und die Weiterentwicklung der Sicherheitstechnik sowie deren Bewertung zur Gewährleistung des sicheren Einschlusses der Abfälle. Die zukünftigen untertägigen Verschlusssysteme mit Bentoniten als Dichtungskomponente übernehmen in diesem Zusammenhang eine zentrale Rolle.

Das beantragte Verbundvorhaben unterstützt die Entwicklung und technische Konzeption von Bentonitdichtungen sowie die Prognose ihrer Langzeitwirkung.

2. Wissenschaftlich-technisches Ergebnis des Vorhabens

Das vorgeschlagene Verbundvorhaben erweitert und vertieft das Verständnis des Langzeitverhaltens von Bentoniten, die als Verschlussmaterial in Schächten in Salzformationen eingesetzt werden sollen.

Es wird die noch fehlende wissenschaftliche Grundlage für die Auswahl möglichst langzeitstabiler Bentonite für den Einsatz in Tonendlagern in Nord-und Süddeutschland (mit hoch- und niedrigsalinaren Porenwässern) geschaffen.

Für den Nachweis der Integrität der geologischen und geotechnischen Barrieren eines Endlagers im Ton sind die mikrobiellen Effekte aufgrund einer möglichen Beeinträchtigung der Barrierewirkung des Wirtsgesteins und der geotechnischen Barrieren zu berücksichtigen. Eine Begrenzung der mikrobiellen Aktivität wird als vorteilhaft bewertet.

3. Fortschreibung des Verwertungsplanes

Die im Rahmen des Projektes gewonnenen Erkenntnisse dienen als Grundlage zur Beurteilung des mikrobiologischen Einflusses auf die Langzeitstabilität des Barriere-Materials Bentonit in Endlager für hoch-radioaktive Abfälle.

Darüber hinaus bilden die im Projekt erzielten Ergebnisse die Grundlage für ein Folgeprojekt UMB II, in der unter anderem die Frage geklärt werden soll, ob die metabolische Aktivität

von Sulfat-Reduzierer – und weiterer Mikroorganismen – einen Effekt auf das Barriere-Material Bentonit haben kann. Ebenso stehen Themen zur Kompaktierung und Gasbildung im Fokus.

4. Arbeiten, die zu keiner Lösung geführt haben

Alle geplanten Arbeiten wurden durchgeführt und erfolgreich zum Ende gebracht.

Die Bestimmung der Zellzahl bzw. Zelldichte und die Verwendung von CARD-FISH Sonden zum Anfärben und quantitativ erfassen bestimmte Mikroorganismengruppen gestaltete sich wie bereits erwartet sehr schwierig und konnte nicht zu adequaten Ergebnissen führen.

Die Gesamt-DNA aus den Bentonitproben konnte nach einem leicht modifizierten Protokoll ,welches ursprünglich von Sonja Selenska-Pobell entwickelten wurde, isoliert werden. Die Amplifizierung der 16S rRNA Gene, deren Sequenzierung und phylogenetische Analyse war allerdings nicht für alle Proben erfolgreich. Aufgrund der geringen Menge an isolierter DNA war eine Real Time Quantitative PCR (oder Q-PCR) leider nicht möglich.

Die Kultivierung von Sulfatreduzierer bzw. Fe(III)-Reduzierer in geeigneten Nährmedien wurde erfolgreich durchgeführt.

5. Präsentationsmöglichkeiten für mögliche Nutzer

Die Ergebnisse der Arbeiten wurden auf internationalen Konferenzen und Workshops vorgestellt und werden in naher Zukunft in wissenschaftlichen Zeitschriften publiziert und somit einem breiten Nutzerkreis zugänglich gemacht.

6. Ausgaben- und Zeitplanung

Das Projekt wurde gemäß dem Arbeitsplan durchgeführt. Der abschließende ausführliche Verwendungsnachweis zusammen mit den Jahresabrechnungen für die Projektlaufzeit wurde dem Projektträger übergeben.

IV. Kurzfassung

Berichtsblatt

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<tr><td rowspan="4">4. Autor(en) [Name(n), Vorname(n)]
Matschiavelli, Nicole
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18. Kurzfassung

1. Derzeitiger Stand von Wissenschaft und Technik

Der Einfluss der Mikroorganismen auf die Umwandlungen im Bentonit ist bislang nur wenig erforscht. Weitgehend unbekannt ist, in welchem Umfang solche Umwandlungen auf molekularer Ebene durch mikrobielle Aktivität beeinflusst werden. Bisherige Arbeiten zu mikrobiellen Effekten in Bentoniten haben sich hauptsächlich mit dem Nachweis des Vorhandenseins von aktiven Mikroorganismen in hoch kompaktierten Bentoniten und der Produktion des korrosionsfördernden Sulfids, jedoch nicht mit Bentonit-Umwandlung auseinandergesetzt. Umfangreiche Laboruntersuchungen mit Tonmineralen und in Nährmedien gezüchteten Kulturen von anaeroben Mikroorganismen – die mit den Bentonit-eigenen Mikroorganismen durchaus identisch sein können – zeigen, dass bis zu 30 % des Fe(III) in der Smektit-Struktur innerhalb von wenigen Wochen mikrobiell – und nur 1 - 2 % abiotisch in den sterilen Kontrollen – reduziert werden können. Diese Reduktion beeinflusst die Smektit-Struktur und kann in einer Smektit-Auflösung und Mineralneubildung resultieren. Sowohl Fe(III)-reduzierende als auch Sulfat-reduzierende Bakterien und Methan produzierende Archaeen, die in Bentoniten und Tongesteinen indigen sind, können diese Reaktion in Gang bringen und halten.

2. Begründung/Zielsetzung der Untersuchung

Ziel der Arbeiten ist die Entwicklung abgesicherter, objektiver Kriterien zur Auswahl geeigneter Bentonite für den Einsatz in Endlagern für wärmeentwickelnde Abfälle in Tonformationen. Durch ein aufeinander abgestimmtes experimentelles und modelltheoretisches Arbeitsprogramm sollen Lücken im Prozessverständnis der Bentonitumwandlung im Kontakt zu Formationslösungen geschlossen werden. Das Vorhaben „UMB“ sollte in vernetzten Einzelprojekten offene Fragen zur Bentonitstabilität klären.
Eine Unsicherheit liefert in diesem Zusammenhang die Mikrobiologie. Reaktionen, die abiotisch nicht stattfinden, sind im Kontakt mit Mikroben möglicherweise als relevant anzusehen, da Mikroben spezielle Reaktionen katalysieren können. Weitgehend unbekannt ist, in welchem Umfang Umwandlungen quellfähiger Montmorillonite im Bentonit durch mikrobielle Aktivität beeinflusst werden. Hierzu wurden Untersuchung des Einflusses mikrobieller Effekte auf die Alteration von Bentoniten durch Reduktion von Fe(III) zu Fe(II) bei 25 °C, 60 °C und 90 °C (Experimente GRS, Mikrobiologische Untersuchungen HZDR) durchgeführt.

3. Methode

Zur Untersuchung des mikrobiologischen Einflusses auf die Alteration von Bentoniten wurde die mikrobielle Diversität in verschieden Ansätzen (verdünnte Gipshutlösung oder Opalinustonporenlösung, Temperaturen 25 °C, 60°C und 90°C, mit Substraten oder ohne) mittels kulturunabhängiger (Isolation von DNA, Amplifikation von 16S rRNA Genen, Sequenzierung und phylogenetische Analyse) und kulturabhängiger (Anreicherung und Isolation in verschiedenen Kulturmedien) untersucht.

4. Ergebnis

Es wurde gezeigt, dass die mikrobielle Diversität und Aktivität stark vom jeweiligen Bentonit und den Umweltbedingungen (verdünnte Gipshutlösung oder Opalinustonporenlösung, Temperaturen 25 °C, 60°C und 90°C, mit Substraten oder ohne) abhängt. Die Untersuchungen zeigten, dass nach ein bzw. zwei Jahren Inkubation bei 25 °C besonders die, mit Substrat versetzten, SD80-Mikrokosmen die Bildung von schwarzen Präzipitaten sowie die Bildung von Gasen bzw. Hohlräumen zeigten und in den entsprechenden SD80-Ansätze Sulfat-reduzierende und Sporen-bildende Bakterien, welche Sulfat als terminalen Elektronen-Akzeptor nutzen können und Schwefelwasserstoff als Reaktionsprodukt bilden, dominierten. In den B36-Ansätzen dominierten jedoch je nach verwendeter Porenlösung und Temperatur, sporen-bildende Mikroorganismen (Opalinuston Porenlösung, 25 °C), thermophile Mikroorganismen (Opalinuston Porenlösung, 60 °C) oder halophile Mikroorganismen (verdünnte Gipshut Lösung, 25 °C). Die Daten zeigen, dass sich die mikrobielle Diversität in den zwei analysierten Bentoniten sehr voneinander unterscheidet und auch unterschiedlich entwickelt.

5. Schlussfolgerung/Anwendungsmöglichkeiten

Die Ergebnisse dieser Arbeit konnten zeigen, dass sich die mikrobielle Diversität in den zwei analysierten Bentoniten sehr voneinander unterscheidet und auch unterschiedlich entwickelt in Anhängigkeit der gegebenen Bedingungen.. Zusammenfassend können diese Ergebnisse einen wertvollen Beitrag zur geeignete Auswahl des Barriere Materials leisten. .Dadurch könnten negative Prozesse, welche in einem Endlager eintreten könnten (z.B. Korrosion, Umkristallisation), vermieden bzw. eingeschränkt werden, um so eine langfristige und sichere Lagerung der Brennelemente zu gewährleisten.

19. Schlagwörter

Mikroorganismen, Bentonite, DNA-Isolierung, Mikrobielle Diversität, Sulfate- und Eisenreduktion, mikrobielle Isolate

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18. Abstract

1. State of the art

The influence of microorganisms on the transformation of bentonite is only poorly explored. Unknown is the extent of such a transformation on a molecular level via microbial activity. Previous work about the microbial effects in bentonite only prove the presence of active microorganisms in highly compacted bentonite and the production of corrosive sulphides, but did not address the transformation of the bentonite. Extensive lab studies with clays and in culture media grown cultures of anaerobic microorganisms – which can be identical to the bentonite-inhabiting microorganisms – showed that up to 30% of the Fe(III) from the smectite was microbial reduced within a few weeks, whereas only 1-2% were reduced in the abiotic sterile controls. This reduction influences the smectite structure and can result in smectite dissolution and reformation of minerals. Fe(III)- and sulphate-reducing bacteria as well as methane producing archaea, which are indigenous in bentonite and clay stones, can initiate and continue this reaction.

2. Aim of this study

Aim of this study is the development of secured, objective criteria for the selection of suitable bentonites that can be used in a repository of heat-generating waste in clay formations. Through a coordinated experimental and model-theoretical work program gaps in the process understanding of bentonite transformation in the contact with formation solutions will be closed. The project „UMB“ will solve open questions regarding the stability of bentonite via networked individual projects. Uncertainty in this context provides the microbiology. Reactions that don´t take place under abiotic conditions, will be of relevance when they are in contact with microorganisms, because microorganisms can catalyse special reactions. Unknown is, to what extent the transformation of swellable montmorillonite in bentonite is influenced through microbial activity. Therefore investigations regarding the influence of microbial effects on the alteration of bentonite via reduction of Fe(III) to Fe(II) at 25°C, 60°C and 90°C were performed (Experiments GRS, microbiological studies HZDR).

3. Methods

To study the microbial influence on the alteration of bentonites the microbial diversity in different batches (diluted cap rock solution, or Opalinus clay pore water solution, temperatures 25 °C, 60°C und 90°C, with or without substrates) via culture-independent (isolation of DNA, amplification of 16S rRNA genes, sequencing and phylogenetic analysis) and culture-dependent (enrichment and isolation in different culture media) was analysed.

4. Results

The results showed that the microbial diversity and activity depends on the respective bentonite and the environmental conditions (dilutes cap rock solution or Opalinus clay pore water, temperatures 25 °C, 60°C und 90°C, with or without substrate).
After one and two years of incubation at 25 °C, respectively, supplemented SD80 microcosms containing Opalinus Clay pore water showed the formation of black precipitates and fissures as well as the dominance of sulphate-reducing and spore-forming bacteria. The detected genera are able to reduce the present sulphate in order to form hydrogen sulphide. In the B36 batches dominated microorganisms depending on the used pore water solution and temperature, e.g. spore-forming organisms (Opalinus Clay pore water, 25°C), thermophilic microorganisms (Opalinus Clay pore water, 60°C) and halophilic microorganisms (diluted cap rock solution, 25°C). Our data show, that the microbial diversity in the analysed two bentonites and, furthermore, the evolution of the respective microbial communities differs significantly from each other.

5. Conclusions

The results of this study showed, that the microbial diversity in the two analysed bentonites differs significantly from each other and that the evolution of the respective microbial communities develop differently depending on the environmental conditions. To sum up, our results can make a valuable contribution to the proper selection of the barrier material. Thereby potential risks (e.g. corrosion, mineralogical changes), due to microbial activity within the repository can be prevented and/or reduced.

19. Key words

Microorganisms, Bentonite, DNA-isolation, microbial diversity, sulphate- and iron-reduction, microbial isolates

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1. Contents

2. Zusammenfassung

Für die tiefengeologische Lagerung von Wärme-entwickelnden, hoch-radioaktiven Abfällen kommen Bentonite aufgrund ihrer hohen Quellfähigkeit und ihrer geringen hydraulischen Leitfähigkeit als geo-technische Barriere in Betracht, welche sich zwischen der technischen Barriere (Behälter mit Abfall) und der geologischen Barriere (Wirtsgestein) befindet. Im Rahmen des Verbundprojektes „UMB“ (Umwandlungsmechanismen in Bentonitbarrieren) der Kooperationspartner Gesellschaft für Anlagen- und Reaktorsicherheit (GRS) mbH (Fachbereich Endlagersicherheitsforschung), der Universität Greifswald (Institut für Geographie und Geologie), der Bundesanstalt für Geowissenschaften und Rohstoffe (BGR, Arbeitsbereich Technische Mineralogie), der Technischen Universität München (TUM; Fachgebiet Theoretische Chemie, Quantenchemie) und dem Helmholtz-Zentrum Dresden Rossendorf (HZDR Institut für Ressourcenökologie) sollen abgesicherte, objektive Kriterien zur Auswahl geeigneter Bentonite für den Einsatz in Endlagern für wärmeentwickelnde Abfälle in Tonformationen entwickelt werden. Das HZDR analysierte hierfür die Entwicklung der mikrobiellen Diversität in den Bentoniten B36 und SD80 in Abhängigkeit von verschiedenen Parametern (Porenlösung, Temperatur, Anwesenheit von Substraten) um den möglichen Einfluss von Mikroorganismen auf die Umwandlungsprozesse im Bentonit zu erfassen. Die Bentonite wurden hierfür bei der GRS (Gesellschaft für Anlagen- und Reaktorsicherheit gGmbH) mit Opalinuston-Porenlösung bzw. verdünnter Gipshut-Lösung versetzt. Die Ansätze inkubierten in gasdichten Glasflaschen bei 25 °C, 60 °C und 90 °C für jeweils ein und zwei Jahre („Langzeit“). Des Weiteren wurden am HZDR B36 Mikrokosmen mit Opalinustonporenlösung angesetzt, welche für drei Monate bei 30 °C inkubierten („Kurzzeit“). Über die drei Monate verteilt wurden sechs Probenahmen durchgeführt, und die mikrobielle Diversität sowie ausgewählte geochemische Paramater bestimmt.

Nach ein bzw. zwei Jahren Inkubation bei 25 °C zeigten besonders die, mit Substrat versetzten, SD80-Mikrokosmen die Bildung von schwarzen Präzipitaten sowie die Bildung von Gasen bzw. Hohlräumen. Die Analyse der 16S rRNA Gensequenzen der entsprechenden SD80-Ansätze zeigten die Dominanz Sulfat-reduzierender und Sporen-bildender Bakterien, welche Sulfat als terminalen Elektronen-Akzeptor nutzen können und Schwefelwasserstoff als Reaktionsprodukt bilden. Die Röntgenfluoreszenz (RFA) -Analysen der Universität

Greifswald bestätigten die Abnahme der Sulfatkonzentration in den entsprechenden SD80 Proben. Die mikrobielle Diversität des B36 Ausgangsmaterials unterscheidet sich sehr deutlich von der Diversität des SD80 Bentonits. Ähnlich zu den SD80 Langzeit-Ansätzen veränderte sich auch bei den B36 Ansätzen die mikrobielle Diversität sehr deutlich. Je nach verwendeter Porenlösung und Temperatur, dominierten Sporen-bildende Mikroorganismen (Opalinuston Porenlösung, 25 °C), thermophile Mikroorganismen (Opalinuston Porenlösung, 60 °C) oder halophile Mikroorganismen (verdünnte Gipshut Lösung, 25 °C). Des Weiteren konnte die Opalinuston Porenlösung der Langzeit-Versuche beider Bentonite erfolgreich für Anreicherung von Mikroorganismen genutzt werden. Die Substrat-enthaltenden Kurzzeit-Analysen des B36-Bentonits zeigten nach drei Monaten Inkubation eine ähnliche Entwicklung der mikrobiellen Diversität wie die Langzeit-Versuche. Wieder dominierten Sporen-bildende Mikroorganismen. Des Weiteren konnte in den entsprechenden B36-Ansätzen eine Abnahme der Laktat-Konzentration sowie eine Zunahme der Azetat- und Eisen(II)Ionen-Konzentration nachgewiesen werden. Im Allgemeinen förderte die Anwesenheit von Substraten sowie Temperaturen von 25 °C bzw. 30 °C die Dominanz von Mikroorganismen, welche mehr oder weniger „metabolische Generalisten“ sind und Überdauerungsformen ausbilden können. Extreme Voraussetzungen wie beispielsweise hoch-salinare oder thermophile (60 °C) Bedingungen förderten hingegen die Dominanz von spezialisierten Mikroorganismen. Die Daten zeigen, dass sich die mikrobielle Diversität in den zwei analysierten Bentoniten sehr voneinander unterscheidet und auch unterschiedlich entwickelt. Da besonders zu den extremophilen Mikroorganismen und deren Aktivität im Bentonit noch nicht sehr viel bekannt ist, betonen die hier dargestellten Ergebnisse die Wichtigkeit mikrobieller Analysen (mikrobielle Diversität, metabolische Aktivität) im Hinblick auf das Barriere-Material Bentonit. Durch eine geeignete Auswahl des Barriere Materials, könnten so negative Prozesse, welche in einem Endlager eintreten könnten (z.B. Korrosion, Umkristallisation), vermieden bzw. eingeschränkt werden, um so eine langfristige und sichere Lagerung der Brennelemente zu gewährleisten.

3. Summary

Concerning the deep geological disposal of high-level radioactive waste (HLW), bentonite can be used because of its high swelling capacity and its low hydraulic conductivity as geo-technical barrier and buffering material in between the waste-containing canister (technical barrier) and the surrounding host rock (geological barrier). There are still many gaps in process understanding of bentonite transformations, especially in dependence of different temperatures and pore waters. Within the joint-project UMB ("Umwandlungsmechanismen in Bentonitbarrieren"), the co-operation partner Gesellschaft für Anlagen- und Reaktorsicherheit (GRS) mbH (Repository Safety Analysis), the University of Greifswald (Institute for Geography and Geology), the Federal Institute for Geosciences and Natural Resources (BGR, section of technical mineralogy), the Technical University of Munich (TUM; chair of theoretical chemistry, quantum chemistry) and the Helmholtz-Center Dresden-Rossendorf (HZDR, Institute of Resource Ecology) are supposed to define criteria which facilitate the selection of suitable bentonites in order to use them in the deep geological repository of high-level radioactive waste. HZDR analyzed two different bentonites (B36 and SD80) regarding their microbial diversity and potential microbial activity. In dependence of repository-relevant parameters (temperature, pore water, presence of substrates), microcosm experiments were set up at the GRS, containing the respective bentonites and Opalinus Clay pore water or cap rock solution, respectively. The long-term batches were incubated one year and two years at different temperatures (25 °C, 60 °C and 90 °C) in gastight bottles. Additionally, HZDR set up B36 short-term microcosms with Opalinus Clay pore water, which incubated for three month at 30 °C with six sampling points monitoring the microbial diversity and geochemical parameters.

After one and two years of incubation at 25 °C, respectively, supplemented SD80 microcosms containing Opalinus Clay pore water showed the formation of black precipitates and fissures as well as the dominance of sulfate-reducing and spore-forming bacteria. The detected genera are able to reduce the present sulfate in order to form hydrogen sulfide. XRF spectroscopy analysis, done at the University of Greifswald, showed a decrease in sulfate concentration in the respective SD80 microcosms, supporting this surveillance. Similar observations were made for the two-year incubations. The microbial diversity of the

B36 bentonite raw material is much different from the SD80 bentonite raw material. Similar to the diversity of SD80 bentonite, the microbial community of the B36 bentonite long-term incubations changed with respect to the applied pore water. Spore-forming organisms dominated the set ups which were supplied with Opalinus Clay pore water solution whereas halophilic microorganisms were found in set ups containing diluted cap rock solution. We were also successful in showing the dominance of thermophilic bacteria in Opalinus clay pore water-containing microcosms that incubated at 60 °C for two years. Additionally, we were able to enrich microorganism from Opalinus Clay pore water of both, B36 and SD80 bentonite long-term incubations. Similar to the long-term analysis, substrate-containing B36 short-term microcosms, containing Opalinus Clay pore water, showed also the dominance of spore-forming bacteria after three months of incubation. Furthermore, a slight decrease in lactate-concentration as well as an increase in ferrous iron and acetate-concentration was observed in the respective B36 microcosms. The presence of substrates and mesophilic incubation temperatures of 25 °C or 30 °C, respectively, promoted the growth of “microbial generalists” that are able to exist in a vegetative state. Extreme environmental conditions as elevated temperatures (60 °C) or high-salt concentrations promote the dominance of highly specialized microorganisms. Our data show, that the microbial diversity in the analyzed bentonites and, furthermore, the evolution of the respective microbial communities differs significantly from each other. Since not that much is known about intrinsic extremophilic microorganisms (metabolic activity and potential influence on the bentonite barrier material), our data stress the importance of further microbial investigations in order to prevent and reduce potential risks (e.g. corrosion, mineralogical changes), due to microbial activity within the repository.

4. Introduction and Objectives

For the long-term disposal of high-level radioactive waste (HLW), a multi-barrier system is currently the most favored concept. It consists of the natural, geological barrier (host rock), a buffering and sealant material and the metal canister containing the HLW.[1,2] Because of their mineral composition (bentonites consist mainly of montmorillonite), bentonites are characterized by a high swelling capacity and a very low hydraulic conductivity which make them act as a kind of seal, retarding water to contact the steel-container.[3,4] In addition, bentonites have the ability to adsorb and immobilize selective metals and radionuclides,[1,5] hindering the migration of the respective compounds which can be released due to corrosion of the metal canister. Since bentonites and their properties are very diverse, a number of different aspects need to be considered when selecting a suitable, repository-relevant bentonite. Kaufhold and colleagues studied intensively with the focus on the characterization of bentonites.[3,6–9] They specified key issues that are dependent on the concept employed for each individual repository.[10] Important specifications as hydraulic conductivity, gas permeability, stability against iron corrosion and swelling capacity were characterized and compared. However, there are still many gaps in process understanding, especially regarding transformations of bentonite when being in contact with pore water. Different parameters like temperature, pH, redox potential and salinity could have effects on the desired properties of the selected bentonite. Additionally, microbial activity can potentially influence the mentioned parameters and, thus, the properties of selected bentonites. Therefore, it is important to elucidate and to understand these effects in order to define criteria, which are important when choosing best suitable bentonites for the application in the distinct disposal programs.

Microbial activity is not considered to be a major concern and any bacterial growth is likely to be limited by the high swelling pressure of the bentonite seal.[11] However, the bentonite will be in direct contact with the metal canister containing the HLW and, in the long-term, not all areas may remain compacted, the potential for microbial metabolic activity within any selected sealant material should be considered. It is known that microorganisms are present in bentonites[12] and that they can interact with minerals[13], potentially affecting the properties of smectitic clay minerals[13,14,15,16] as well as the adsorption of metals and

actinides *via* a number of processes including the mobilization and immobilization of toxic elements and radionuclides.[17,18,19] Furthermore, microbial influenced corrosion (MIC) may lead to the mechanical failure of metals and alloys used at the HLW repository site.[14,20,21]

The major goal of the joint research project UMB is the development of confirmed, objective criteria for the selection of suitable bentonites for the storage of heat-generating radioactive waste in a deep geological clay formation. Therefore, 15 bentonites were investigated according to their interactions with diluted cap rock solution and Opalinus clay pore water solution at 25°C, 60°C and 90°C and the effect on mineralogical-chemical changes on the hydromechanical properties (swelling pressure and permeability development). Two of these bentonites were selected to investigate the microbial influence on the transformation of the bentonite depending on the addition of substrates, different temperatures as well as repository-relevant pore water solutions. These investigations were performed at the HZDR. In order to evaluate microbial effects on the bentonite barrier material as function of temperature, pore water and the availability of substrates, it is relevant to analyze the present microbial community and their evolution. The obtained results show, that the two analyzed bentonites contain different microbial communities and that they evolve in a different manner, even under the same incubation conditions. Especially spore-forming microorganisms were present and often dominated the microcosms, independent from the bentonite that was applied. In general, elevated temperature, high-salt concentrations and the absence of substrates seemed to prevent a massive microbial growth within the analyzed bentonites. Our results clearly show that microorganisms are present and that they evolve under the applied conditions. Furthermore, our enrichments demonstrate, that intrinsic microorganisms can be “reactivated” and that they have the potential to be metabolically active, providing the potential to change their environment and, thus, properties of the bentonite barrier. Since anaerobic metabolism is always connected with the formation of gases and organic acids, generally used barrier materials can be potentially influenced in their properties due to the formation or consumption of microbial formed metabolites. Therefore, it is important to consider microorganisms prior to the planning and construction of a repository.

5. Materials and Methods

5.1. Bentonites

The processed B36 (Liskovec, Slovakia; Ca-dominated) and SD80 (Milos, Greece; Ca/Mg-dominated) bentonites, used in this study, were provided by Stephan Kaufhold (BGR; Bundesanstalt für Geowissenschaften und Rohstoffe, Hannover). Table 1 shows the respective mineral composition that was analyzed and provided by the University of Greifswald.

Table 1: Mineral composition of B36 and SD80 bentonite. *XRD quantification by Rietveld refinement (BGMN): R_{WP} = weighted residual square sum; R_{exp} = possible minimum value for R_{WP}

Mineral content	B36 [wt-%]	SD80 [wt-%]
smectite	65.9	89.7
feldspars	14.7	6.8
calcite	n.d.	1.4
quartz	12.5	0.6
barite	n.d.	0.6
pyrite	n.d.	0.5
cristobalite	3.7	n.d.
trdymite	1.4	n.d.
chlorite	1.2	n.d.
anatase	0.6	0.5
Rwp*	3.26	3.73
Rexp*	2.20	2.34

n.d.: below detection limit

A detailed characterization of the B36 was done by Kaufhold and colleagues.[9] The Bentonite SD80 was analyzed in this study by the GRS and the University of Greifswald. The two bentonites differ in a number of properties, e.g. swelling capacity, cationic exchange capacity and the type of mining and processing, respectively. The SD80 bentonite e.g. was “sun-dried”, so the temperature did not exceed 100 °C. For further information regarding the mineralogy of the two analyzed bentonites, we recommend the final reports of the GRS and the University of Greifswald.

5.2. Long-term experiments

5.2.1. Setup of long-term batch-experiments

The setup of experiments was performed by the "Gesellschaft für Anlagen- und Reaktorsicherheit" (GRS, Braunschweig; Figure 1). For this purpose 300 g of the respective bentonite material (B36 or SD80) was supplied with 600 ml synthetic Opalinus Clay pore-water solution (212 mM NaCl, 26 mM $CaCl_2$, 14 mM Na_2SO_4, 1.6 mM KCl, 17 mM $MgCl_2$, 0.51 mM $SrCl_2$ and 0.47 mM $NaHCO_3$)[22] or saturated NaCl-$CaSO_4$ cap rock solution (2.52 M NaCl, 10 mM $CaCl_2$, 8 mM Na_2SO_4, 5.1 mM KCl) and filled up in glass flasks which were welded after filling. For the stimulation of microbial processes, some batches were supplied with a mixture of organics (50 mM acetate, 50 mM lactate, 3 mM methanol and 0.1 mM AQDS).

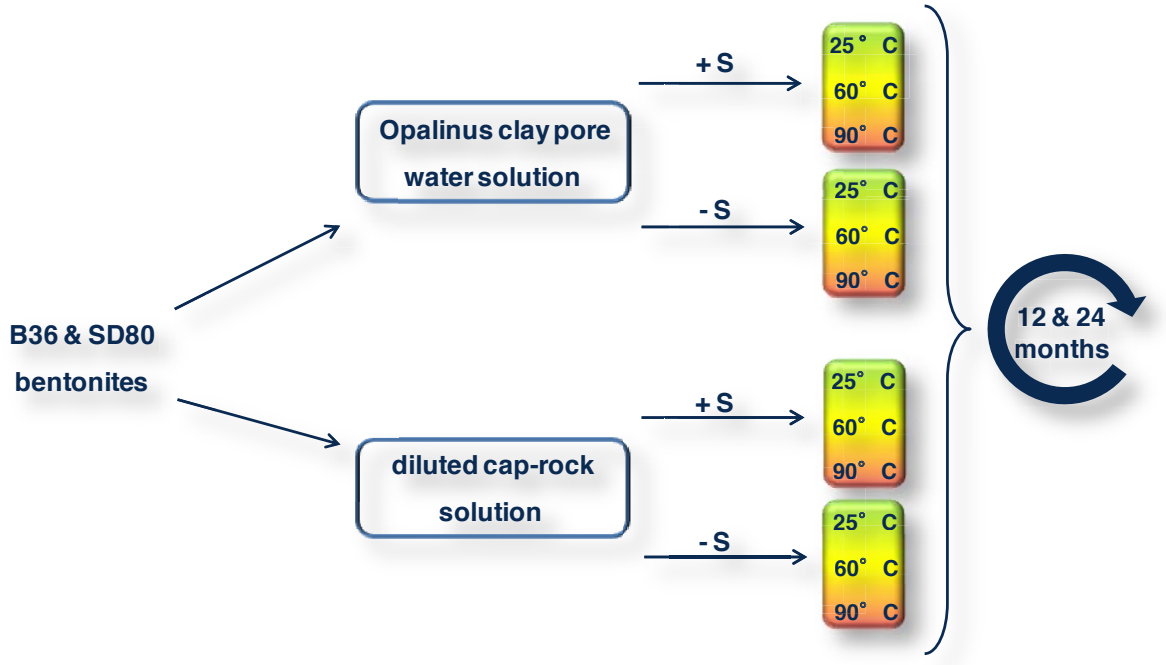

Figure 1: Schematic overview of long-term batch-experiments setup by GRS. S: Substrate (50 mM lactate, 50 mM acetate, 3 mM methanol and 0.1 mM AQDS).

The whole experiment was setup in an unsterile manner. The batch-experiments incubated one year and two years at 25 °C, 60 °C and 90 °C in the dark, without shaking at the GRS. After one year and two years, respectively, samples were taken and analyzed regarding their microbial, geochemical and mineralogical development, whereas the latter ones where carried out at the GRS and University of Greifswald, respectively. For further information regarding the set up, we refer to the final report of the GRS.

5.2.2. Sampling of batch-experiments for analyzing microbial diversity

The sampling of batch-experiments after one year and two years of incubation at the respective conditions, took place in the laboratories of the GRS in Braunschweig. At first, samples were taken for the enrichment of microorganisms and for analyzing the microbial diversity. Therefore, the respective flasks were opened by a glass blower and sampled under a fume hood. With a sterile and disposable syringe up to five ml of pore water supernatant from the respective microcosm were transferred into prepared media (see 5.2.3). Afterwards, an undefined amount of the respective bentonite slurry was taken with a disposable and sterile spatula and transferred into two sterile 50 ml tubes. Each tube was screwed tightly and sealed with Parafilm®. The slurry samples were stored at -70 °C until extraction of DNA.

5.2.3. Cultivation and Enrichments

For the enrichment and cultivation of microorganisms from long-term microcosm-experiments, one or two ml of incubated microcosm supernatant was transferred with disposable and sterile syringes into supplemented media. Prior to use, the syringes were

Table 2: Composition of basic medium for the enrichment and cultivation of anaerobic microorganisms from incubated microcosm experiments. Shown is the required amount for the production of 1 l medium.

compound	**amount [l]**
K_2HPO_4	0.3
KH_2PO_4	0.2
NH_4Cl	0.3
$MgCl_2$ x 6 H_2O	0.4
$CaCl_2$ x 2 H_2O	0.1
NaCl	12.4 g/146.1 g*
MOPS-buffer solution (1 M, pH 7.2; 50 X)	20 ml
Trace element solution (1000 X)	1 ml
$NaHCO_3$	3.8 g
Vitamin solution (500 X)	2 ml
Yeastextract	0.1 g
Resazurin solution (1000 X)	1 ml

*: For enrichments originating from Opalinus Clay water containing microcosms, 12.4 g NaCl were used whereas 146.1 g NaCl were used for enrichments and cultivation of microorganisms originating from microcosms supplied with diluted cap rock solution.

flushed several times with N_2 gas in order to ensure an anaerobic transfer. The composition of the used basic medium is depicted in Table 2. Cells were enriched and cultivated in 26 ml-serum tubes[23] (Ochs, Bovenden, Germany) containing 5 ml or 10 ml and 100 ml-serum bottles (Ochs) containing 30 ml of the respective enrichment-medium. The composition of the used trace element- and vitamin- stock solutions as well as the final concentration of the respective components in the media, are shown in Table 3 and Table 4, respectively.

Table 3: Composition of trace element solution.[24] Given are the amounts for a 200 ml stock solution (1000 X) and the final concentration in the medium.

components	amount [g/200 ml stock solution]	final concentration [µM]
Nitrilotriacetic acid	2.22	58
$FeSO_4 * 7 H_2O$	1.134	20
$NaSeO_3 * 5 H_2O$	0.578	11
$CoCl_2 * 6 H_2O$	0.2	4
$MnSO_4 * H_2O$	0,2	6
$Na_2MoO_4 * 2 H_2O$	0.2	4
$Na_2WO_4 * 2 H_2O$	0.2	3
$ZnSO_4 * 7 H_2O$	0.2	3
$NiCl_2 * 6 H_2O$	0.6	12
H_3BO_3	0.02	1.6
$CuSO_4 * 5 H_2O$	0.02	0.4

Table 4: Composition of vitamin solution.[24] Given are the amounts for a 200 ml stock solution (500 X) and the final concentration in the medium.

components	Amount [mg/200 ml stock solution]	final concentration [nM]
4-Aminobenzoate	10	729
Nicotinic acid	10	812
D-Pantotheic acid (hemicalcium salt)	10	419
Pyridoxine hydrochloride	10	486
Riboflavin	10	266
Thiamine hydrochloride	10	296
Biotin	5	204
Folic acid	5	113
alpha-Lipoic acid	5	242
Cobalamine	5	37

The basic media was combined and transferred into a stood round-bottom flask made of glass. The media was flushed with a permanent stream of N_2/CO_2 gas (80:20 [v/v]) while stirring for one hour (one liter medium) and two hours (two to three l medium). Afterwards, the flushed medium was introduced into an anaerobic glove box (MB-200B modular glove box workstation; M. BRAUN; Garching, Germany) containing an N_2-atmosphere. The medium was supplemented with 1 g Cysteine hydrochloride and 0.05 g Na_2S for complete reduction. After complete reduction (color change of added Resazurin from blue over pink to colorless), the medium was filled up into serum glass bottles and balch tubes and closed with a butyl rubber stopper and an aluminum shell (Ochs, Bovenden/Lenglern). Subsequently, the media was taken out of the box, the gas atmosphere was defined with 40 kPa N_2/CO_2 (80:20 [v/v]) and the media was autoclaved. The ready-made medium was supplemented with different combinations of electron donors and electron acceptors as well as inhibitors selective against methanogens (2-Bromoethanesulphonic acid, BES)[25,26] and Bacteria (Streptomycin sulfate).[27] The whole spectra of used supplements is shown in Table 5.

Table 5: Overview of applied electron donors and acceptors used for the selective enrichment of microorganisms from microcosm experiments.

electron donor	**electron acceptor**	**carbon-source**	**selectivity**
Methanol	-	Methanol/CO_2	-
Methanol	-	Methanol/CO_2	Streptomycin
Methanol	-	Methanol/CO_2	BES
H_2	CO_2	CO_2	-
H_2	CO_2	CO_2	Streptomycin
H_2	CO_2	CO_2	BES
Lactate	SO_4^{2-}	Lactate/CO_2	-
Glucose	Glucose	Glucose	-

For individual selection of microorganisms, supplements were added from sterile, anaerobic stock solutions (Table 6).[28] All solutions were flushed with N_2 gas for 30 minutes while stirring. The respective solutions of methanol, lactate, Na_2SO_4 and BES were afterwards autoclaved. The solutions of glucose and streptomycin sulfate were filtrated (pore size of 0.2 µm) into anaerobic and sterile glass flasks.

Table 6: Electron donors and –acceptors used for the selective enrichment of microorganisms from microcosm experiments. All supplements were applied in an sterile and anaerobic manner into the media.

supplements	**anaerobic stock solution**	**final concentration in media**
Methanol	6.25 M	62.5 mM
Na_2SO_4	1 M	25 mM
Glucose	1 M	25 mM
Lactate, sodium salt	0.5 M	25 mM
2-Bromoethanesulphonic acid (BES)	0.5 M	5 mM
Streptomycin sulfate salt	10 mg/ml	100 µg/ml

The bottles were incubated at 30 °C in the dark. Growth and purity of enriched cultures was monitored and controlled with an Olympus BX61 transmitted and reflected light microscope (Thalheim Spezialoptik GmbH, Pulsnitz, Germany).

5.2.4. Extraction of DNA

For analyzing the microbial diversity within the respective samples, DNA was extracted using a protocol from Selenska-Pobell[29] with the following adjustments: step 1: centrifugation of samples for 15 min at 6,800 x g; step 2: for removal of SDS, centrifugation speed was adjusted to 11,000 x g; step 3: addition of PEG-6000 to a final concentration of 40 % with a centrifugation speed set at 11,500 x g. For purification and concentration of extracted genomic DNA, NucleoBond® AXG 100 columns and recommended buffers (G4, N2, N3 and N5) were used following the instruction manual. Genomic DNA was then precipitated by using 0.4 volumes of isopropanol (molecular grade). After sedimentation of DNA at 12,000 x g, it was resolved in a TE-buffer.

5.2.5. Amplification of 16S rRNA genes and sequencing

The extracted DNA was utilized for amplification of the V4-region of the 16S rRNA gene by using PCR.[30] The PCR reactions contained 5 µl PCR water, 1 µl $MgCl_2$ (2.5 mM final concentration), 2 µl 5x buffer (Promega), 0.4 µl of each oligonucleotide (0.4 µM final concentration), 0.1 µl dNTPs (125 µM final concentration), 0.1 µl Taq-Polymerase (Promega GoTaq G2 Flexi, 0.05 U/µl final concentration) and 1 µl genomic DNA or appropriate

dilutions. For amplification of the V4 region, oligonucleotides 515f (GTGCCAGCMGCCGCGGTAA) and 806r (GGACTACHVGGGTWTCTAAT)[31] were used. Reactions were held at 95 °C for 2 min to denature the DNA, with amplification proceeding for 30 cycles at 95 °C for 30 s, 50 °C for 60 s and 72 °C for 60 seconds. A final step of 10 min at 72 °C was added to the procedure to ensure complete amplification. The successful amplification of DNA was checked *via* gel electrophoresis. Successfully amplified DNA was purified with MSB® Spin PCRapace (Stratec molecular) according to the manufacturer`s protocol. Purified amplicons were quantified and the respective quality was controlled by using a NanoDrop™ (Thermo Scientific™) and following the manufacturer`s protocol. The samples were sequenced with MiSeq Illumina at RTL Genomics (Texas, USA). Data analysis was undertaken following the guidelines of RTL Genomics (www.rtlgenomics.com/docs/Data_Analysis_Methodology.pdf).[32]

5.3. Short-term experiments

5.3.1. Microcosm Experiments and Sampling

Microcosm-experiments were set up by placing 20 g of the respective bentonite powder into sterile glass bottles and subsequent addition of 40 ml sterile, anaerobic synthetic Opalinus Clay pore-water solution (212 mM NaCl, 26 mM $CaCl_2$, 14 mM Na_2SO_4, 1.6 mM KCl, 17 mM $MgCl_2$, 0.51 mM $SrCl_2$ and 0.47 mM $NaHCO_3$, degassed with a N_2/CO_2 gas mixture (80/20) while stirring).[22] Selected microcosms were supplemented with sterile and anoxic 50 mM acetate, 50 mM lactate, 0.1 mM methanol and AQDS (Anthraquinone-2,6-Disulfonate). Control microcosms included sterilized bentonite (autoclaving for 20 min at 121 °C and 1 bar) or only the pore-water without the addition of bentonite. The bentonite was not compacted and there was no additional pressure applied. Microcosms were incubated for 98 days at 30 °C in the dark without shaking. During the incubation period, samples were taken at six different time-points and analyzed for bio-geochemical parameters and microbial diversity. For each time point and each condition, duplicates were set up. The sampling was carried out under strictly anaerobic conditions in an anaerobic glove box (MB-200B modular glove box workstation; M. BRAUN; Garching, Germany) containing an N_2-atmosphere. Microcosms were introduced into the glove box, well mixed, and a portion of the suspension (10 ml) transferred by pouring it into sterile 50 ml tubes for geochemical analyses. Since

freezing and storage at -70 °C is widely considered suitable for preserving microbial composition,[33] we stored the remaining suspension in the resealed bottle at this temperature for no longer than two months after sampling prior to DNA-extraction. For iron-determination, 300 µl samples taken from the well-mixed suspension were placed in 1.5 ml tubes and treated as described below. Sensory measurements of O_2-concentration, redox potential and pH were performed by using calibrated sensors. The centrifuged and filtered (0.2 µm filter) supernatant of the suspension was used for determining the concentration of organic acids and sulfates.

5.3.2. Determination of Ferric and Ferrous Iron, Sulfate and Organic Acids

Ferric and ferrous iron were determined using a modified protocol of Voillier *et al.*[34] Approximately 300 µl of well-mixed microcosm suspension were added to 12.4 µl of 12 M HCl. The samples were incubated in the dark for 30 minutes at room temperature within an anaerobic glove box. Afterward, the HCl-suspension-mix was centrifuged and 200 µl of the clear supernatant (or appropriate dilutions in 212 mM NaCl solution) applied to the ferrozine-assay. The colorimetric test was conducted as described[34] and relative values of ferric and ferrous iron calculated by comparison with standard solutions prepared as dilutions of 2 mM $FeCl_3$ in 10 mM HCl (starting from 5 µM up to 100 µM).

Organic acids were identified *via* HPLC analysis with an "Agilent 1200" (Degasser G1322A, diode array detector G1315B, quaternary pump G1354A). For separation, a Nucleogel Ion 300 OA column was used. The releasing agent was 5 mM H_2SO_4 with a flow rate of 0.4 ml/min at 70 °C. For the quantification of organic acids, adequate dilutions of sodium acetate, sodium lactate and sodium pyruvate were used. Dilutions were prepared in synthetic Opalinus Clay pore-water.

The sulfate-concentration was determined by ion chromatography using a Dionex Integrion HPIC (Thermo Scientific). For calibration, a K_2SO_4-standard solution was used (0.05 mg - 10.0 mg SO_4^{2-}) and, like the samples, separated by utilizing an AS23-column (Thermo Scientific). A defined Na_2CO_3-H_2CO_3 mixture served as eluent with a flow rate of 250 µl/min.

5.3.3. Extraction of DNA, Amplification of 16S rRNA genes and sequencing

The DNA was extracted as described in Chapter 2.2.3. The extracted DNA was utilized for amplification of the V1-V3-region of the 16S rRNA gene by using PCR.[30] The PCR reactions contained 5 µl PCR water, 1 µl $MgCl_2$ (2.5 mM final concentration), 2 µl 5x buffer (Promega), 0.4 µl of each oligonucleotide (0.4 µM final concentration), 0.1 µl dNTPs (125 µM final concentration), 0.1 µl Taq-Polymerase (Promega GoTaq G2 Flexi, 0.05 U/µl final concentration) and 1 µl genomic DNA or appropriate dilutions. For amplification of the V1-V3 region, oligonucleotides 519r (GTNTTACNGCGGCKGCTG) and 28f (GAGTTTGATCNTGGCTCAG)[35] were used. Reactions were held at 95 °C for 2 min to denature the DNA, with amplification proceeding for 25 cycles at 95 °C for 30 s, 50 °C for 60 s and 72 °C for 60 seconds. A final step of 10 min at 72 °C was added to the procedure to ensure complete amplification. The successful amplification of DNA was checked *via* gel electrophoresis. Successfully amplified DNA was purified with MSB® Spin PCRapace (Stratec molecular) according to the manufacturer`s protocol. Purified amplicons were quantified and the respective quality was controlled by using a NanoDrop™ (Thermo Scientific™) and following the manufacturer`s protocol. The samples were sequenced with MiSeq Illumina at RTL Genomics (Texas, USA). Data analysis was undertaken following the guidelines of RTL Genomics (www.rtlgenomics.com/docs/Data_Analysis_Methodology.pdf).[32]

6. Results and Discussion

6.1. Microbial diversity in SD80 bentonite long-term incubations

In order to analyse the effects of temperature, pore water and the presence of substrates on the microbial diversity of microorganisms present in SD80 bentonite, long-term microcosms were set up by GRS. The DNA of all 25 samples (including SD80 raw material) was successfully extracted (Table 7). The complete 16S rRNA gene analysis is available for two long-term experiments (Table 7).

Table 7: Amount of extracted DNA and sequencing result from SD80 bentonite long-term setups. Shown are the concentrations of isolated DNA and the result of the amplified and sequenced V4-region of 16S rDNA.

T	SD80 samples	DNA [ng/µl] (1 year)	16S rRNA gene sequencing	DNA [ng/µl] (2 years)	16S rRNA gene sequencing
	O	3.1	–	3.5	–
25 °C	**O + S**	89.9	+	297.2	+
	C	1.1	–	5.3	–
	C + S	38.4	–	4.7	–
	O	6	–	7	–
60 °C	**O + S**	15.4	–	10.4	–
	C	2.7	–	4.7	–
	C + S	3.8	–	4.3	–
	O	2	–	3.1	–
90 °C	**O + S**	3.1	–	4.6	–
	C	1.8	–	1.7	–
	C + S	3.4	–	3.7	–

O: synthetic Opalinus Clay pore water, C: diluted caprock solution, S: includes supplements (organics), T: Temperature, +: extracted DNA was successfully sequenced; -: no sequence available.

In general, the DNA content of the majority of the SD80 microcosms was very low. A successful amplification and sequencing of the 16S rRNA genes was only realized for samples that were incubated for one year and two years at 25 °C with Opalinus Clay pore water supplemented with substrates. The DNA concentration of the respective samples was comparably high (Table 7) and the corresponding SD80 bentonite showed the formation of black precipitates and spots as well as the formation of gases (Figure 2 and Figure 3).

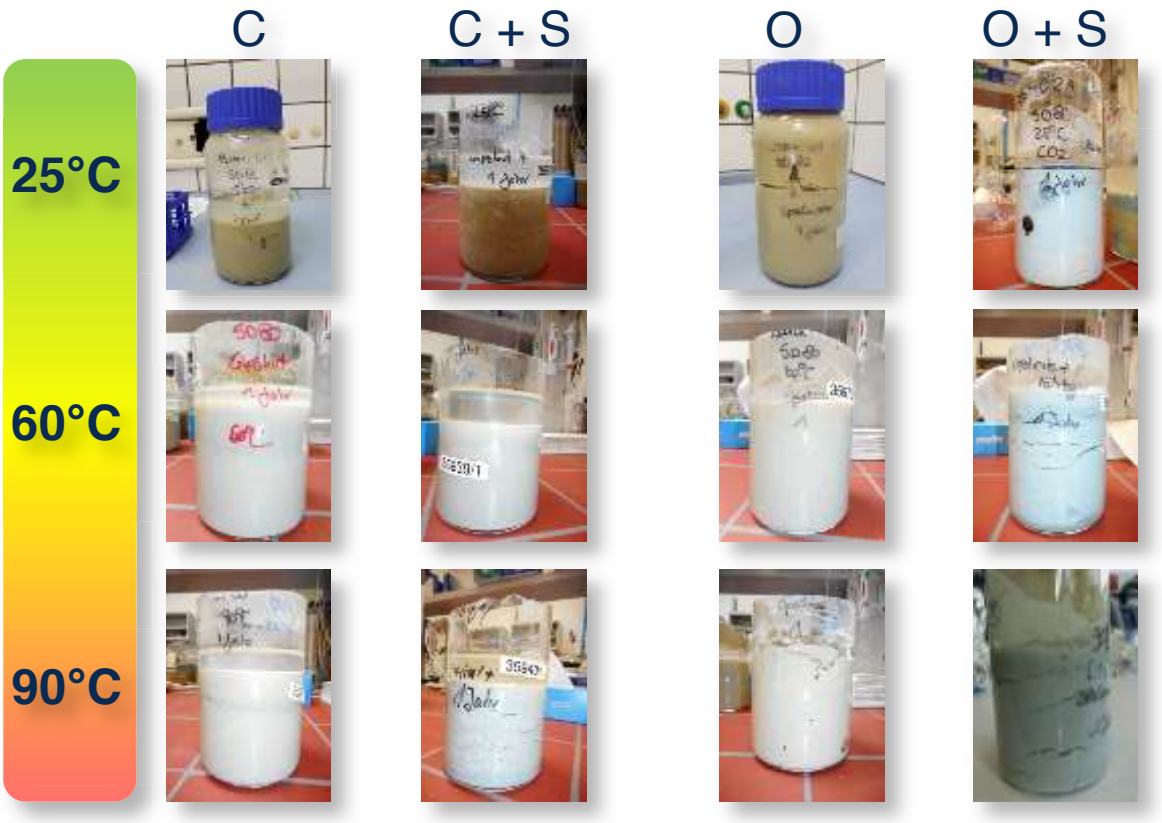

Figure 2: SD80 Microcosms after one year incubation at the respective temperatures. C: diluted cap rock solution; O: Opalinus Clay pore water; S: Substrate (50 mM lactate, 50 mM acetate, 3 mM methanol and 0.1 mM AQDS).

The SD80 raw material showed typical soil organisms including some genera known to form spores (Figure 4). Microcosms that were incubated at 25 °C with supplemented Opalinus Clay pore water, showed a significant change in their microbial diversity compared to the raw material. After one year of incubation under the respective conditions, microcosms were dominated by sulfate-reducing bacteria from the genus *Desulfitobacterium* (up to 50 %). Further sulfate-reducing and spore-forming bacteria that were detected in the respective microcosm belong to the genera *Desulfosporosinus* (8 %) and *Desulfotomaculum* (8 %, Figure 4). After two years of incubation under the same conditions, the microcosm was clearly dominated by species from the genus *Desulfosporosinus* (60 %, Figure 4). In general, the conditions in these setups were selective for sulfate-reducing organisms, namely the presence of lactate and sulfate, the mesophilic temperature of 25 °C and no oxygen-supply. Thus, the here detected genera, are better adapted to the mentioned conditions compared to those, that have been detected in the SD80 raw material. The changing dominance of microorganisms in the respective batches could be due to different handling or processing

during the set up (one-year and two-year experiments were set up at different time points) or simply due to the inhomogeneous distribution of the respective genera in the supplied bentonite-material. However, the growth of these microorganisms leads to an increase of biomass, which may explain the higher yield of DNA.

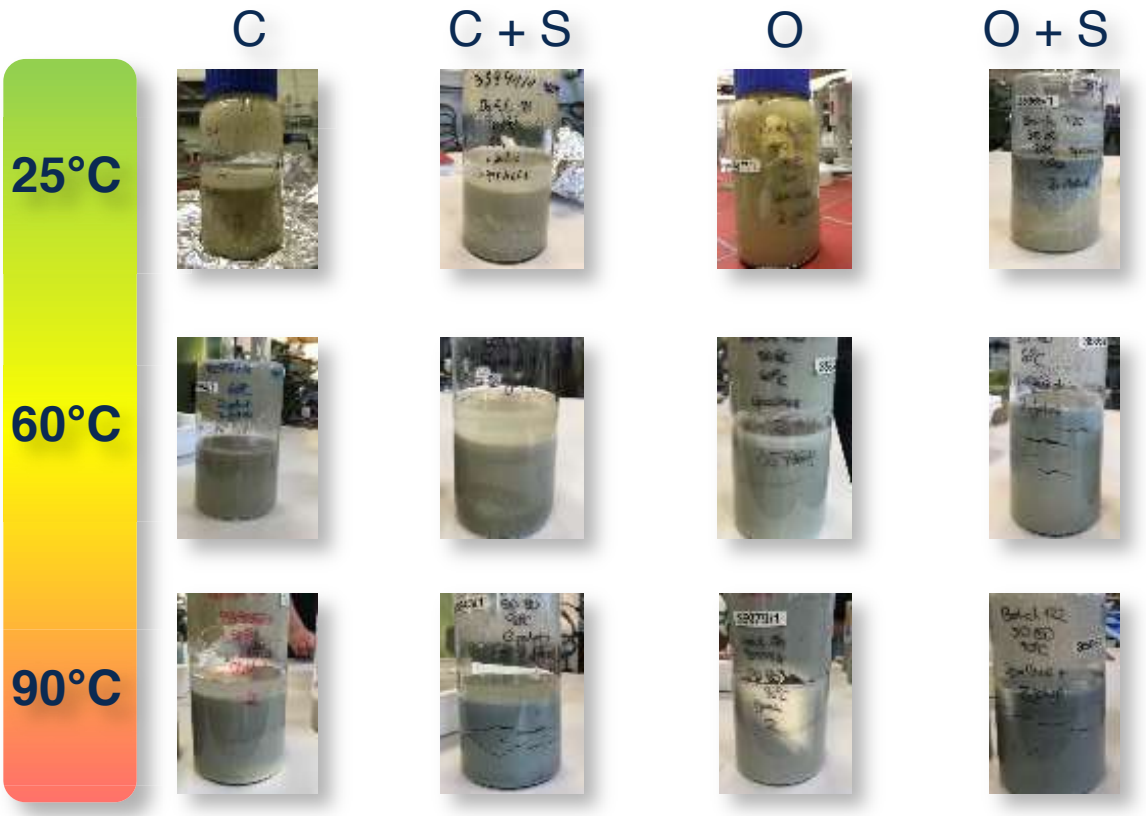

Figure 3: SD80 Microcosms after two years incubation at the respective temperatures. C: diluted cap rock solution; O: Opalinus Clay pore water; S: Substrate (50 mM lactate, 50 mM acetate, 3 mM methanol and 0.1 mM AQDS).

Generally, the here applied conditions seemed to be selective for sulfate-reducing bacteria which can use the supplied lactate as energy source, thereby using sulfate as terminal electron-acceptor.[36] As a result, hydrogen sulfide is formed which is known to promote the corrosion process.[37,21] The mentioned sulfate-reducing bacteria have been also detected in MX-80 bentonite, demonstrating that their presence is of relevance in bentonites.[38,39,11] The finding of a decreased sulfate concentration *via* XRF spectroscopy by the University of Greifswald (personal communication with C. Podlech), supports the thought, that they were metabolically active in the respective SD80 set ups.

The formation of the already mentioned gases and black precipitates, which is indicative for the formation of insoluble iron sulfides, also points to the generation of hydrogen sulfide. This thought is also supported with analyses done by the GRS, showing that the concentration of ferrous iron increased in supplemented SD80 microcosms that incubated at 25 °C (personal communication A. Meleshyn). This iron reduction could be due to the microbial formation of hydrogen sulfide gas. The reduction of smectitic ferric iron to ferrous iron could lead to a potential destabilization of the smectite and, therefore, to a loss of its swelling and sealing function.[40,41] This sulfide-mediated iron reduction could also explain the observed increase of ferrous iron and simultaneous decrease of ferric iron as well as the observed formation of grey precipitates, probably indicative of iron-sulfur particles.

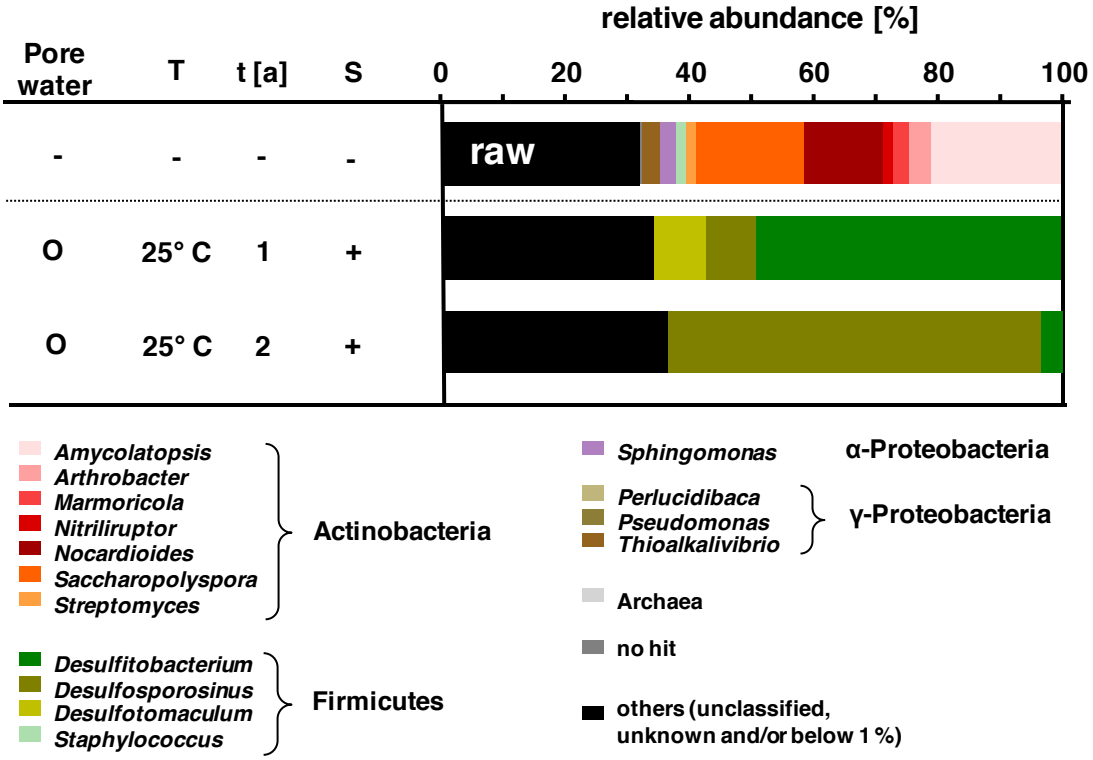

Figure 4: Microbial diversity in SD80 samples. Shown is the relative abundance of detected genera and their dependence on the added electron donors (S: lactate, acetate, methanol and AQDS) and incubation time (t in days [d]). The microbial community was analyzed by amplifying and sequencing the V4-region of the 16S rRNA gene *via* MiSeq Illumina. C: diluted cap rock soulution; O: Opalinus Clay pore water.

Further mineralogical analysis of the University of Greifswald showed, that the cationic exchange capacity (CEC) of SD80 microcosms, that incubated with substrate-containing Opalinus Clay pore water at 25 °C, are lower compared to CEC-data of SD80 raw material (personal communication with C. Podlech). Since CEC values are dependent from many

factors including solvent chemism, dilution- and precipitation processes as well as the presence of organic acids, it has to be elucidated in further experiments to what extend this and further parameters are influenced due to microbial activity.

A high number of 16S rRNA gene sequences (up to 30%) was assigned to unknown or unclassified Bacteria, representing an unknown metabolic potential within the SD80 bentonite and, thus, potential influences on the bentonite, which we might not have in mind, yet. Thus, further analysis would be necessary in order to analyze these microbial communities in more detail.

However, we were successful in cultivating microorganisms from one-year SD80 microcosms that incubated at 30 °C. Morphological similar microorganisms were observed in enrichments that were supplemented with methanol and streptomycin, with lactate and sulfate and with methanol and BES (Figure 5; A, B and C).

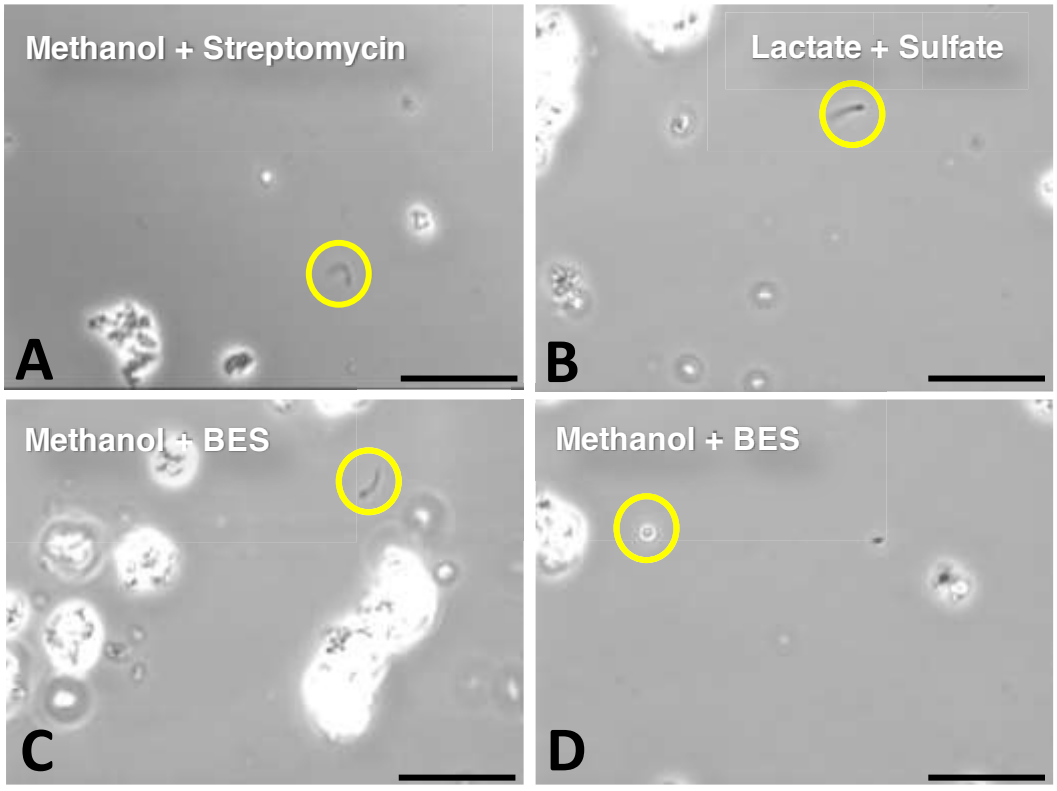

Figure 5: Light microscopic photographs from enrichments of SD80 bentonite long-term incubations. For inoculation of the enrichments, the supernatant pore water from Opalinus Clay pore water-containing SD80 bentonite microcosms that incubated for one year at 25 °C without substrates was used. The single figures show the presence of microorganisms when transferred into enrichment media containing A: methanol and streptomycin, B: lactate and sulfate, C and D: methanol and BES. The respective enrichments were incubated at 30 °C in the dark. The bar represents 20 µm.

Methanol and streptomycin is generally selective for methylotrophic, methanogenic archaea or methylotrophic bacteria, that are resistant to streptomycin. In combination with BES, methanol serves as energy source for methylotrophic bacteria. Growth of methanogenic archaea is inhibited in these enrichments. Lactate and sulfate are classical substrates for the cultivation of sulfate reducing organisms, as detected and sequenced in supplemented SD80 bentonite long-term incubations.

Since the here observed microorganisms show indications for the formation of spores, like the refracting circle in Figure 5 D and the terminal bud in Figure 4 A, B and D, we cannot rule out that the here observed microorganism is a spore-former, handling all the here applied environmental conditions. So far, we did not analyze these enrichments further. It would be helpful to isolate these microorganisms, in order to use them for further analysis like their influence on swelling pressure, hydraulic conductivity, cationic exchange capacity and, in general, their influence on parameters that are important when choosing the bentonite for the repository. However, the microscopic pictures make clear, that indigenous microorganisms of SD80 bentonite can be activated under several conditions, meaning that these microorganisms bring in the potential to change their environment and thus may influence the composition and mineralogical properties of the bentonite, especially in the long run.

6.2. Microbial diversity in B36 bentonite long-term incubations

For a better understanding of microbial diversity in bentonites and potential metabolic effects, the long-term analysis of B36 bentonite was compared with results obtained with microcosms containing SD80 bentonite. The DNA of all 25 B36 samples (including B36 raw material) was successfully isolated (Table 8). Amplification and sequencing of the 16S rRNA genes was realized for four samples. In general, the DNA concentration of the majority of the B36 microcosms was again very low, independent from the applied growth conditions and incubation time (Table 8). This observation is similar to the results obtained for the SD80 long-term analysis and is indicative for low biomass content in the analysed microcosms.

Table 8: Amount of extracted DNA and sequencing result from B36 bentonite long-term setups. Shown are the concentrations of isolated DNA and the result of the amplified and sequenced V4-region of 16S rRNA gene. ; -: sequencing was not successful.

T	B36 samples	DNA [ng/µl] (1 year)	16S rRNA gene sequencing	DNA [ng/µl] (2 years)	16S rRNA gene sequencing
25 °C	**O**	5.6	–	3.3	–
	O + S	7.9	+	9.9	–
	C	1.7	+	3.8	–
	C + S	4.7	+	3	–
60 °C	**O**	5.8	–	5.8	–
	O + S	7	–	6	+
	C	7.3	–	4.6	–
	C + S	5.4	–	5.3	–
90 °C	**O**	3.4	–	8.2	–
	O + S	5.7	–	6.1	–
	C	0.4	–	3	–
	C + S	2.3	–	6.5	–

O: synthetic Opalinus Clay pore water, C: diluted caprock solution, S: includes supplements (organics), T: Temperature, +: extracted DNA was successfully sequenced; -: no sequence available.

Especially substrate-containing microcosms showed significant changes in bentonite coloration after one year and two years of incubation as well as the formation of fissures and gases (Figure 6, Figure 7).

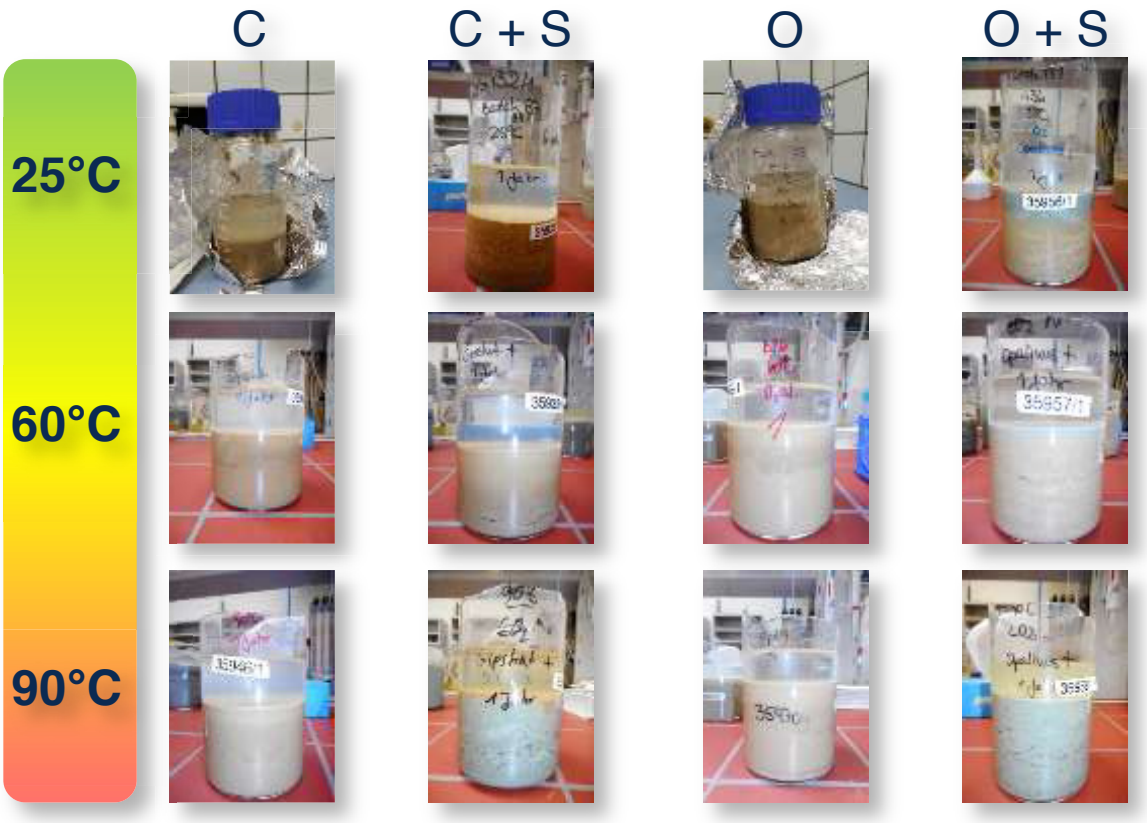

Figure 6: B36 microcosms after one year incubation at the respective temperatures. C: diluted cap rock solution; O: Opalinus Clay pore water; S: Substrate (50 mM lactate, 50 mM acetate, 3 mM methanol and 0.1 mM AQDS).

The microbial diversity of the B36 bentonite raw material was different compared to the SD80 raw material, suggesting that different bentonites contain different microbial communities. This observation can be a result due to different factors e.g. indigenous microbial within the bentonites themselves, mining regions, mining itself, different processing steps and/or (unsterile) handling in general. This means, that with respect to the bentonite that might be used in the repository, different microbial communities will be brought in. This indicates that the microbial diversity and activity and their evolution for B36 or SD80 bentonite, determined in this study, cannot be extrapolated to other bentonites. Thus, every suitable bentonite should be also analyzed for its microbial diversity and, furthermore, for their potential evolution and metabolic activity.

However, raw material of B36 bentonite showed the presence of conventional, mesophilic soil organisms (Figure 8). Some of the here detected genera are metabolically very versatile and are able to form spores (e.g. *Saccharopolyspora*, *Streptomyces* and *Bacillus*).[42,43,44] In general, the formation of spores enables the respective microorganisms to be resistant to

heat, pressure, desiccation, UV and γ-radiation treatments, even if the respective exposure lasts for many years.[45,44,46]

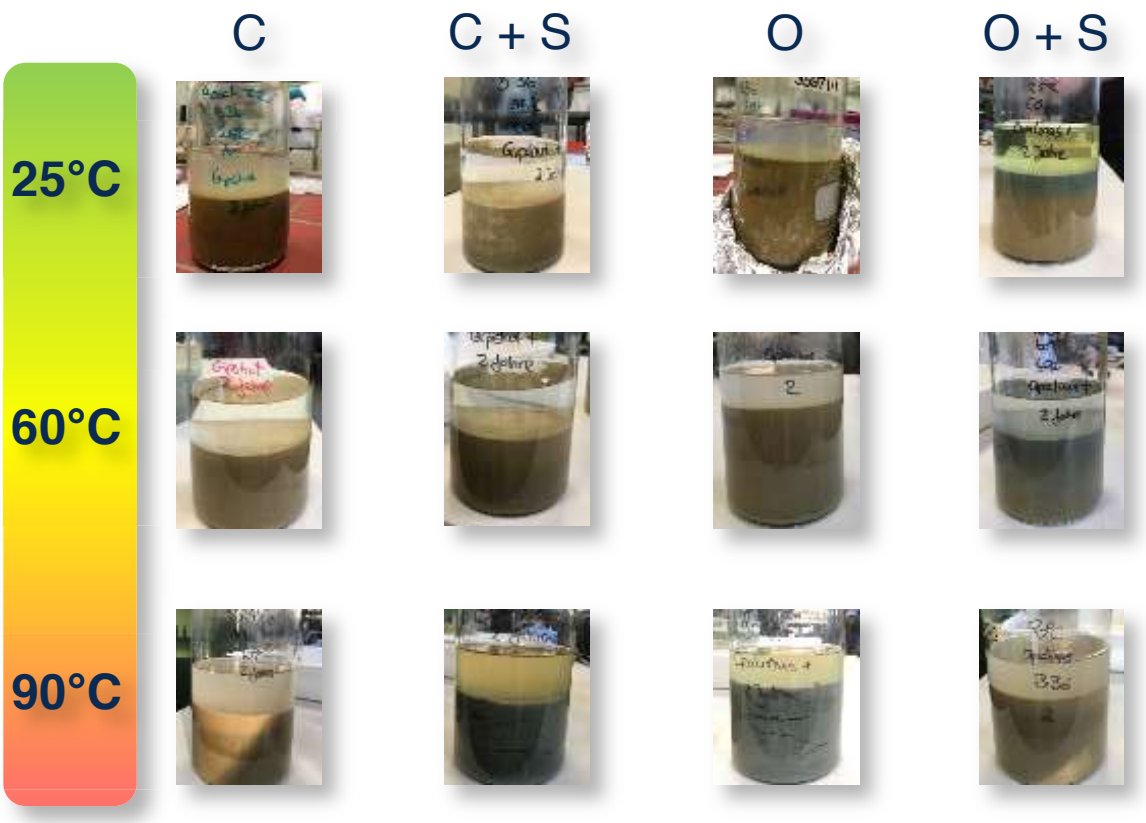

Figure 7: B36 microcosms after two years incubation at the respective temperatures. C: diluted cap rock solution; O: Opalinus Clay pore water; S: Substrate (50 mM lactate, 50 mM acetate, 3 mM methanol and 0.1 mM AQDS).

The mentioned spore-forming microorganisms were still present in microcosms that incubated at mesophilic temperatures when diluted cap rock solution or Opalinus Clay pore water was present. After one year incubation in the microcosm that contained diluted cap rock solution (C+S, 25 °C) the mesophilic genera *Marinobacter*, which comprises species that are known to be extremely halotolerant, dominated the microbial community (Figure 8).[47] Whereas microcosms that were incubated under the same conditions, except the addition of substrates (C, 25 °C), a *Bacillus* species dominated, that is able to form spores and cope with the extreme halophilic conditions.[48] *Bacillus* species dominated also B36 bentonite microcosm experiments that incubated one year at 25 °C in the presence of supplemented Opalinus Clay pore water (O+S, 25 °C; Figure 8). Interestingly, microcosms that incubated for two years at 60 °C with supplemented Opalinus Clay pore water, were dominated by the genera *Symbiobacterium* (O+S, 60 °C, Figure 8). Some representative species of this genus

are known to be thermophilic.[49] Both, *Marinobacter*- and *Symbiobacterium*-dominated microcosms, show distinct changes in coloration or texture of B36 bentonite (Figure 6, Figure 7). To what extend the present/detected microorganisms contribute to these bentonite transformations has to be elucidated in further experiments. Analysis of ferrous iron concentration in supplemented B36 microcosms, showed no significant changes (personal communication A. Meleshyn). Furthermore, CEC measurements by the University of Greifswald showed larger variations in CEC values for the B36 bentonite (personal communication C- Podlech). Similar to SD80 bentonite, a microbial influence in general cannot be ruled out, but further experiments are necessary for addressing their role in more detail.

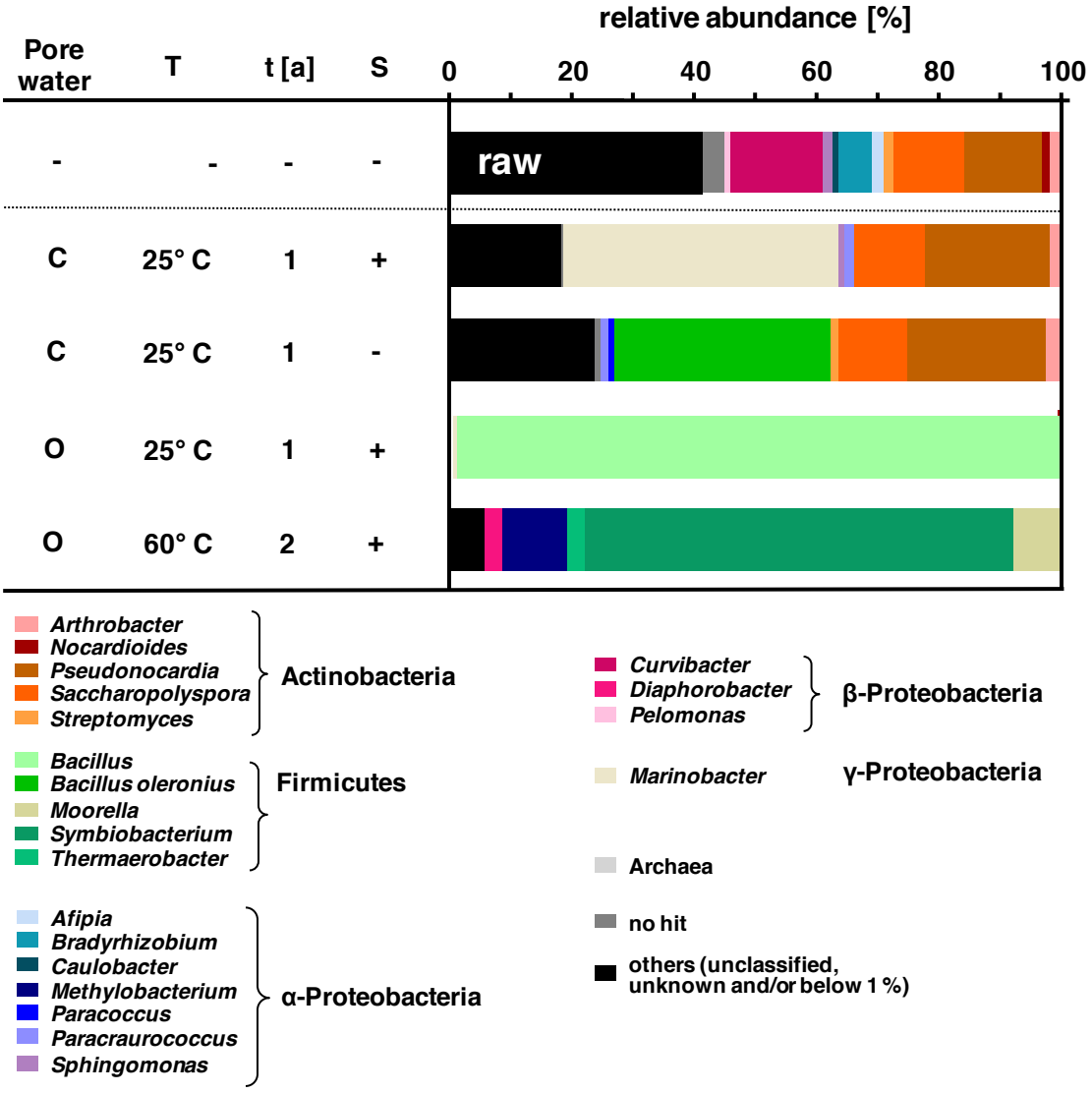

Figure 8: Microbial diversity in B36 samples. Shown is the relative abundance of detected genera and their dependence on the added supplements (S: lactate, acetate, methanol and AQDS) and incubation time (t in days [d]). The microbial

community was analyzed by amplifying and sequencing the V4-region of the 16S rRNA gene *via* MiSeq Illumina. C: diluted cap rock soulution; O: Opalinus Clay pore water.

Again, the changing dominance of microorganisms in the respective batches could be due to various reasons, as already mentioned for the SD80 bentonite. Another important point is the observation that, similar to SD80 bentonite analysis, about 40 % of the analyzed 16S rRNA gene sequences of the B36 raw material were assigned to unknown or unclassified Bacteria, which means that there is the potential of further microbial activity that cannot be addressed yet (Figure 8).

We were also successful in enriching microorganisms from one-year long-term incubations that incubated at 25 °C. Glucose-containing enrichment-media showed the presence of diverse groups of microorganisms showing that glucose serves as energy source for many microorganisms within the B36 bentonite (Figure 11). We did not analyze these enrichments further, yet. But again, similar to observations made for SD80 long-term incubations, the microscopic pictures indicate that indigenous microorganisms of B36 bentonite can be activated under the applied conditions.

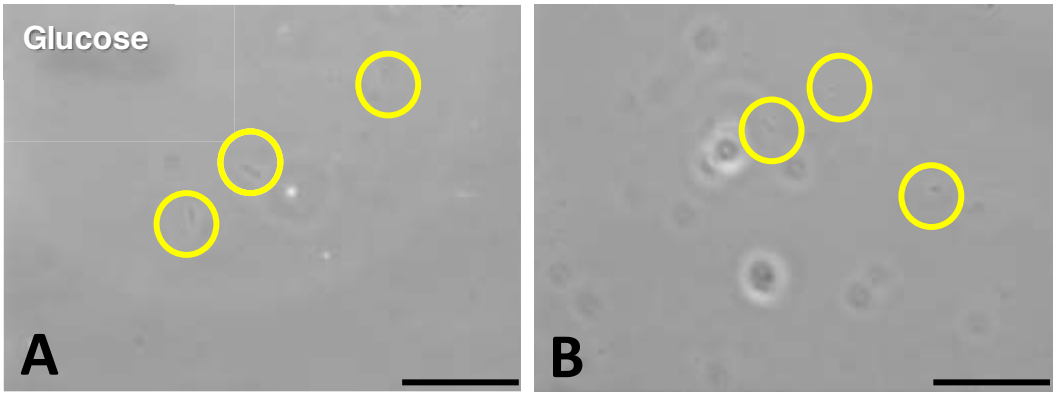

Figure 9: Light microscopic photographs from enrichments of B36 bentonite long-term incubations. For inoculation of the enrichments, the supernatant pore water from Opalinus Clay pore water-containing B36 bentonite microcosms that incubated for one year at 25 °C without substrates was used. The single figures show the presence of diverse microorganisms when transferred into enrichment media containing glucose (A and B). The respective enrichments were incubated at 30 °C in the dark. The bar represents 20 µm.

Thus, the potential of metabolically active microorganisms that can cope with the applied conditions is feasible and should be of relevance in further projects dealing with the characterization of suitable bentonites. Therefore, the determination of metabolites like gases (methane, carbon dioxide, carbon monoxide, hydrogen sulfide, hydrogen) and organic

acids (acetate, lactate, pyruvate, formate, malate) should be monitored in order to draw a connection between the formed and/or consumed metabolites, the present microbial community and resulting mineralogical and geochemical effects on B36 bentonite. Therefore, we set up some short-term B36 bentonite microcosms experiments in order to combine the information about the evolution of the microbial communities with the analysis of some geochemical parameters (see chapter 6.3).

6.3. Bentonite B36 microcosm experiments (short-term incubations)

In order to analyse the metabolic potential and the diversity in B36 bentonite being in contact with synthetic, anaerobic Opalinus Clay pore water solution, we set up microcosms and analysed not only the microbial diversity but also different geochemical parameters as e.g. pH, redox potential, sulfate concentration as well as iron concentration and the concentration of organic acids over a period of 98 days at 30 °C. The results showed that the indigenous microbial community in the analysed microcosms changed even within this short time of incubation. The detected genera were mostly spore-forming bacteria belonging to

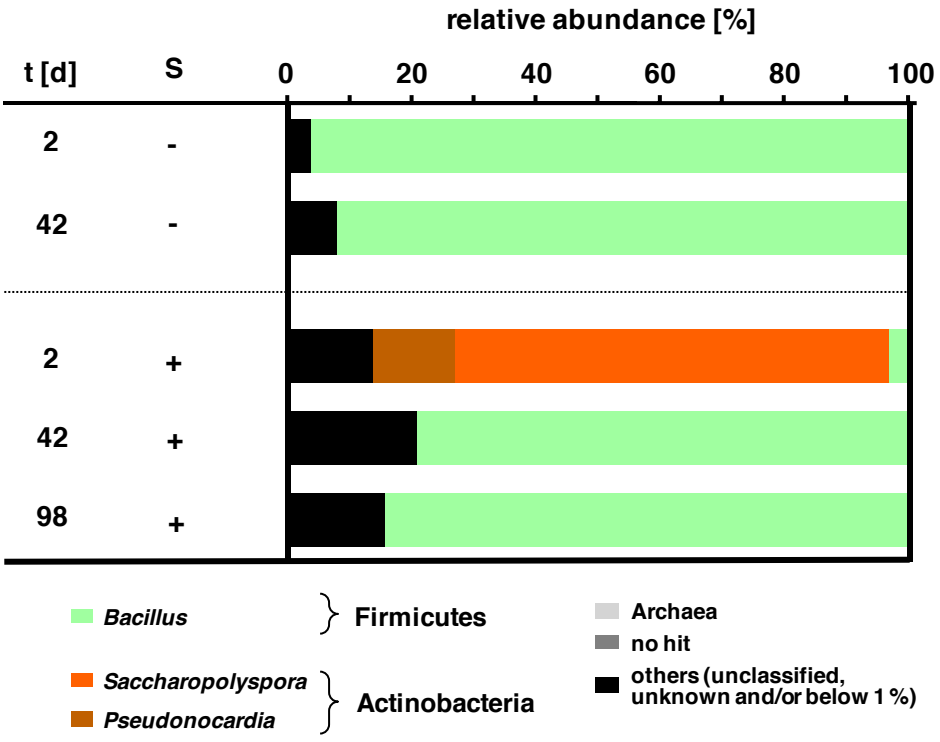

Figure 10: Microbial diversity of selected B36 bentonite microcosms that were incubated at 30 °C with synthetic Opalinus Clay pore water. Shown is the relative abundance of detected genera and their dependence on the added supplements (lactate, acetate, methanol and AQDS) and incubation time (t in days [d]). The microbial community was analyzed by amplifying and sequencing the V1-V3-region[35] of the 16S rRNA gene *via* MiSeq Illumina.

the genera *Bacillus* and *Saccharopolyspora* (Figure 10), which were also dominant in the long-term incubations of the B36 bentonite at 25 °C (see chapter 6.2).

After 98 days of incubation, B36 microcosms showed a green coloration of the bentonite material as well as a green coloration of the supernatant pore water (Figure 11).

Figure 11: B36 microcosms containing supplemented Opalinus Clay pore water after 98 days incubation at 30 °C.

So far, we do not know what exactly the reason for this colour change is. Mineralogical analysis by the University of Greifswald, using XRD, revealed no significant changes in mineralogy (data not shown). Thus, further analysis is necessary to analyse and evaluate the respective observations. Furthermore, the analysis of organic acids showed a decrease in lactate- and an increase in acetate-concentration, as well as the formation of pyruvate (Figure 12), indicating that the present microorganisms are able to metabolize the provided carbon sources. Microbial excretion of pyruvate has been observed before[50,51,52]. Since pyruvate is a very valuable metabolite for every organism, excretion is here very likely due to a stress reaction, caused by the high lactate concentration within the microcosms compared to starved microorganisms within the raw bentonite. As former analysis showed in similar set ups, lower concentrations of organics can be used in order to promote microbial activity.[53] Similar observations regarding the pyruvate formation have been made for starved *E. coli* cells, showing excretion of pyruvate due to inactive pyruvate dehydrogenase[52]. The detected genus *Saccharopolyspora* is known to metabolize organics and *Bacillus* species in general are known to be very versatile regarding their energy source.[42,54] Thus, further

investigations are necessary in order to speculate what metabolic processed took place in the respective samples and if these microbial processes caused the changed coloration of the bentonite. Thus, the use of single organic acids in lower concentrations might be useful to analyse the microbial potential in more detail.

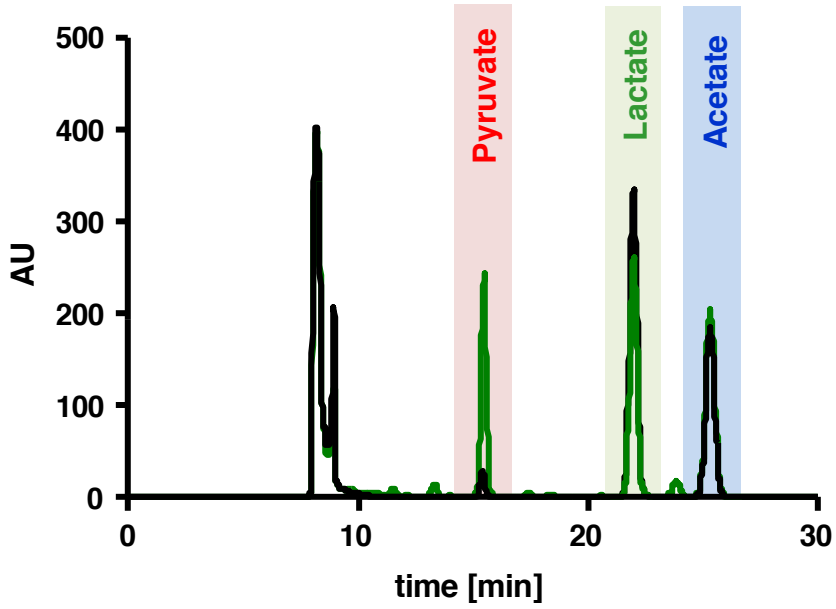

Figure 12: HPLC-chromatogram of filtrated microcosm-supernatant after 2 days (black line) and after 98 days (green line).

Furthermore, substrate-containing microcosms showed significant changes in geochemical and biological parameters. The redox potential decreased from 505 mV to 270 mV whereas the pH remained stable (Figure 13, A). The redox potential has a big influence on the solution behaviour of minerals and ions, thus, an accelerated decrease in redox potential, due to microbial activity might influence these processes much more. Strikingly, an increase of ferrous iron was monitored whereas ferric iron decreased (Figure 13, B). This result needs further experimental analysis in order to understand the observed process. Biotic processes, namely a direct reduction of ferric iron as well as an indirect reduction *via* the supplied electron shuttle AQDS due to microbial activity, is just as possible as abiotic processes.[55,56,57] The changing iron concentrations might be also an explanation for the observed colour-change. But to strengthen this correlation, more in-depth mineralogical analyses are a need.

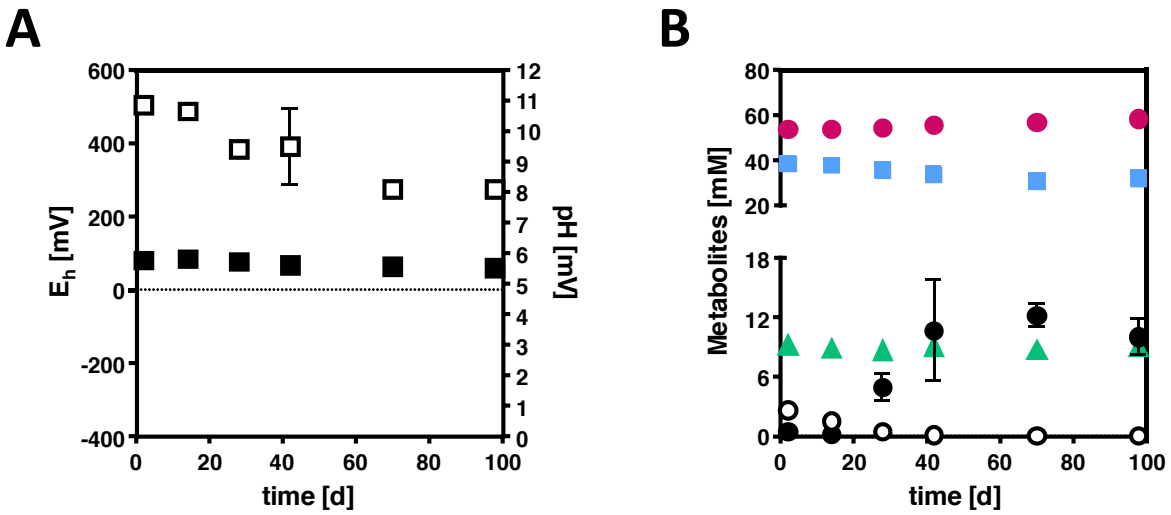

Figure 13: Change of geochemical parameters and metabolites recorded in B36 bentonite microcosms. The setups were incubated in the presence of synthetic Opalinus Clay pore-water solution at 30 °C for 98 days. Shown are the results from microcosms containing acetate, lactate, methanol and AQDS. Panel A shows the change in redox potential (E_h;□) and pH (■) whereas in panel B the concentration of lactate (■), acetate (●), sulfate (▲), HCl-extractable ferric iron (○) and HCl-extractable ferrous iron (●) are depicted. Shown are mean values with standard deviations measured on two independent microcosms.

However, the obtained results clearly demonstrate that theses mentioned processes could play a relevant role in bentonite transformation and should be given an adequate consideration in further investigations.

7. Conclusions

The microbial diversity analysis of this study clearly indicates that the two here analyzed bentonites B36 and SD80 contain intrinsic microorganisms. Long-term as well as short-term analysis showed, that microorganisms are present and that the microbial diversity changes with respect to the applied conditions. To realize this, the respective genera must have been metabolically active. Independent from the used bentonite, temperature, pore-water and the presence of substrates clearly influence the microbial diversity and the evolution of indigenous microorganisms. Our results show, that the detected genera can adapt to high-salt concentrations and to elevated temperature, as we were able to detect dominant species of thermophilic and halophilic microorganisms in the respective microcosms. Furthermore, we did first approaches in enriching microorganisms from the long-term microcosm-experiments, showing that indigenous microorganisms are viable and can be “re-activated” when environmental conditions are more favorable. Since the repository should

be, save and stable for at least 300,000 years,[58] a changing environment, more suitable for intrinsic microorganisms, is feasible.

Here, it should be also mentioned that the analyzed microcosms incubated under ideal conditions of no compaction with the availability of water and energy sources. Nevertheless, no significant high amounts of DNA could have been isolated. Thus, the conditions within the different microcosms were very likely too harsh for a substantial increase in biomass of indigenous microorganisms. In general, elevated Temperatures (60 °C and 90 °C) as well as the absence of substrates and high salt concentrations, as present in microcosms containing diluted cap rock solution, are restrictive to many microorganisms, but provide also the opportunity to well adapted microorganisms, called extremophiles, like the here detected thermophilic and halophilic bacteria. The formation of spores enables the here identified sulfate reducers to outlast during harsh conditions. When being active, these bacteria are able to significantly change their environment – the bentonite barrier – due to a general exchange of ions with their surrounding environment. Data from the GRS and the University of Greifswald support this assumption as changes in iron concentration and CEC were reported. However, further analyses are necessary to address the microbial role in changing the mentioned parameters and, thus, potential properties of the bentonite itself.

To elucidate the microbial metabolic potential within bentonites and to evaluate potential impacts on the applied material(s), it is necessary to measure and quantify metabolites (e.g. the formation and consumption of organic acids and gases) that are produced due to metabolic activity as shown in the B36 microcosms (short-term incubations). Due to corrosion processes hydrogen gas will be formed, which is known to fuel whole microbial communities.[59] The addition of single substrates (hydrogen gas, lactate or acetate) to bentonite slurries with artificial pore water, also in combination with the addition of copper or steel plates, would be helpful to interpret microbial diversity and activity much more, especially regarding their influence on the container material. This is of interest especially regarding their influence of used materials in the final repository. Furthermore, the measurements will be more significant, when simulating conditions that will be in a repository right after closure – namely pressure and relevant temperatures.

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10. Reports and Publications

- Poster: „*Microbial transformation of bentonite*“ ; N. Matschiavelli, J. Steglich, S. Kluge, A. Cherkouk; 16th International Clay Conference - Clays, from the oceans to space (17. - 21.07.2017 in Granada, Spain
- Poster: „*Microbial transformation of bentonite*“ ; N. Matschiavelli, J. Steglich, S. Kluge, A. Cherkouk; GDCh-Wissenschaftsforum Chemie 2017 Interdisziplinäre Symposien Jahrestagung Nuklearchemie, 10 - 14.09.2017, Berlin, Germany
- Poster: “Bentonite – geotechnical barrier and source for microbial life**”** N. Matschiavelli, J. Steglich, S. Kluge, and A. Cherkouk; VAAM-DGHM 2017, Würzburg, Germany
- Report: “Bentonite – geotechnical barrier and source for microbial life**”** N. Matschiavelli, J. Steglich, S. Kluge, and A. Cherkouk; Annual report 2016; Institute of Resource Ecology, page 57
- Report: „Evolution of microbial diversity in bentonite-microcosms“; N. Matschiavelli, S. Kluge, V. Prause, A. Meleshyn and A. Cherkouk; Annual report 2017; Institute of Resource Ecology, page 54
- Oral presentation: “Microbial Influence on Bentonite Transformation”; N. Matschiavelli, J. Drozdowski, S. Kluge, T. Arnold and A. Cherkouk, UMB annual project meeting; GRS Braunschweig, 02.06.2016
- Oral presentation: “Microbial Influence on Bentonite Transformation”; N. Matschiavelli, J. Drozdowski, S. Kluge, T. Arnold and A. Cherkouk, UMB annual project meeting; GRS Braunschweig, 22.09.2017
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11. List of tables

12. Illustration directory

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